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IMPERIAL AGRICULTURAL
RESEARCH INSTITUTE NEW DELHI

H14IARI—5,000— 25-5-46— GIES

CANADIAN JOURNAL OF RESEARCH

VOLUME 20

1942

SECTION C



CANADA

Published by the
NATIONAL
RESEARCH COUNCIL
of CANADA

19223

content, but neither it nor the methods in which oxidizing agents were used were sufficiently superior to the simple 15-min. boiling and aeration treatment to warrant their continued use.

The pH of sulphite waste liquor after removal of the free sulphur dioxide by 15 min. of boiling is 2.80 to 2.90. In order to have satisfactory media for the growth of most fungi, this must be raised to approximately 5.0. This can be accomplished by the addition of bases, such as calcium, barium, magnesium, sodium, and ammonium carbonates and hydroxides, sodium bicarbonate, and calcined brucite. Sulphite liquor media neutralized with these substances alone, or in combination, were tested for suitability in so far as growth of fungi was concerned. Brucite, calcium, magnesium, sodium, and ammonium carbonates were selected for subsequent experiments in which quantitative determinations of pH change and of sugar and lignin losses were made.

Sulphite waste liquor is deficient in nitrogen and phosphorus, both of which are essential for the growth of fungi. It is therefore necessary to add optimum amounts of these in a form in which they are readily available. The following nitrogen compounds were tested: ammonium sulphate, nitrate, carbonate, monohydrogen phosphate, dihydrogen phosphate, potassium nitrate, casein, peptone, several amino acids, and urea. The phosphorus was usually added in the form of phosphate, although very good results may be obtained with phosphoric acid. The sources used were sodium and potassium mono- and dihydrogen phosphates, phosphoric acid, and magnesium glycerophosphate. All of these were satisfactory, but the ammonium phosphates had the advantage of supplying nitrogen in the same compound and keeping the salt content of the media at the lowest level. Mono- and dihydrogen phosphates alone or in combination were used in order to obtain desired pH levels.

Methods and Analytical Procedure

In most of the experiments the raw liquor was boiled for 15 min. with vigorous aeration to remove sulphur dioxide. The neutralizing agents were added to the hot solution and the mixture allowed to stand overnight. The supernatant liquor was poured off and filtered through "Supercel" on a Buchner funnel. If the pH was not satisfactory, further neutralizing material was added and the mixture refiltered. One hundred ml. amounts of this solution were pipetted into 500 ml. Erlenmeyer flasks and sterilized for 20 min. at 120° C. The necessary nutrients, which had been separately sterilized, were added after cooling.

The organism used in these experiments was *Endoconidiophora adiposa* (Butler) Davidson, a wood staining fungus which grew rapidly and produced a smooth surface mat. Culture flasks received an inoculum of 1 ml. of a spore suspension of the fungus and the controls 1 ml. of sterile water.

The flasks were incubated in two large water baths in which the temperature was accurately controlled to within $\pm 0.1^\circ$ C. and the humidity of the

air was high enough to prevent undue evaporation during the growth period. Difficulty was experienced with contaminating organisms growing down through the cotton plug of the flask. This was overcome by igniting the cotton plug as soon as the cultures were sterilized and immediately capping it with a waxed paper drinking cup.

Triplicate pairs of control and inoculated flasks were randomized as to position in the bath. The analysis included measurements of pH, reducing sugars, and calcium lignosulphonate.

The pH was determined by means of a Beckmann pH meter using a glass electrode. Reducing sugars were determined by the method outlined by Partansky and Benson (9).

A survey of the available methods for the analysis of calcium lignosulphonate indicated that the method devised by Hägglund *et al.* (4) and later modified by Partansky and Benson (9) was most suitable. Some changes were introduced. The essential difference between the procedure used and that outlined by Partansky and Benson was the use of 15 ml. of β -naphthylamine for the precipitation, as it was found that 10 ml. of reagent was not sufficient to produce as complete precipitation of the calcium lignosulphonate as was possible with the larger amount. Additional amounts did not produce more precipitation. The second difference was the shortening of the air drying of the precipitate from 12 to 24 hr. to one hour, and the using of the oven drying period of three to four hours. Any loss in volume due to evaporation during the incubation period was taken into account in the final calculation.

The results obtained by using the modified procedure were uniform and consistent enough to warrant its use for the determination of relative lignin content of the samples used in these experiments. Fundamentally, it cannot be considered a method that yields absolute values because the precipitate of β -naphthylamine lignosulphonate is not of constant composition and the factors by which it is converted into terms of lignin are open to criticism.

Experimental

1. EFFECT OF GROWTH PERIOD AND INCUBATION TEMPERATURE ON LIGNIN DECOMPOSITION

The first experiment was designed on data already accumulated from qualitative experiments in which satisfactory growth of the fungus had been obtained. Several different fungi were tested and *Endoconidiophora adiposa* was chosen because it gave rapid growth and produced a firm felty mat on the surface of the medium and could be readily separated from the medium before analyses were made. The length of growth period, and the effect of two different temperatures, were the factors investigated in the first quantitative experiments. The liquor was treated with 5 gm. per litre of calcium carbonate and magnesium carbonate, respectively, to neutralize the acids. The pH was 7.29. After autoclaving, 5 ml. of a 2.4% solution

of ammonium monohydrogen phosphate was added to each flask. Eighteen inoculated and 18 control flasks were placed in each of the two water baths held at 25° and 30° C. Analyses were made at the beginning of the experiment, and at 5, 10, 20, 35, and 70 day intervals for determination of pH change and sugar and lignin losses. The results are shown in Table I.

TABLE I

THE EFFECT OF TEMPERATURE AND LENGTH OF GROWTH PERIOD ON THE DECOMPOSITION OF SULPHITE WASTE LIQUOR BY *Endoconidiophora adiposa*

Growth period, days	Temperature, 25° C.				Temperature, 30° C.			
	pH		Sugar fermented, %	Lignin decomposed, %	pH		Sugar fermented, %	Lignin decomposed, %
	Controls	Test			Controls	Test		
0	6.21	6.21	0.00	0.00	<div>19223</div> <div>■■■■■■■■■■</div> <div>IARI</div>		0.00	
5	6.27	6.01	14.51	0.23				
10	6.10	6.29	46.98	1.42				
20	4.49	6.40	82.03	1.73				
35	5.12	6.90	84.93	8.11				
70	5.47	7.50	82.39	7.79				
							0.00	
							0.00	
							0.96	
							4.90	
							5.39	
							9.31	

There was a small initial difference in the pH values between the flasks at 25° and 30° C. The pH values showed some irregularities, but the general trend was downward in the controls and upward in the inoculated flasks. The downward trend in the controls was probably due to the slow oxidation of the sulphur compounds to sulphuric acid. The initial drop in pH in the cultures was due to the accumulation of carbon dioxide and organic acids produced in the fermentation. After 10 days this stage was over and the pH increased.

Almost 50% of the fermentable sugars are removed in 10 days and 80% in 20 days. Since yeast is able to utilize only 60% of the total sugars, it is apparent that *E. adiposa* is more efficient in this respect.

The lignin in the control flasks did not remain constant but underwent a steady apparent increase up to about 20 days and then became stabilized. The order of the increase was 3 to 4% of the weight of calcium lignosulphonate in solution. This increase was calculated as the difference between the lignin content of the uninoculated control at the beginning of the experiment and at any other time during the course of the experiment. Since the loss in lignin could not be determined as the difference between the amount in solution at the beginning of the experiment and the amount present in the inoculated solutions at any time during the experiment, it was estimated as the difference between an inoculated and control solution as found at any time during the growth period. The lignin loss in 35 days at 25° C. was about 8%. At 70 days there was no further breakdown of the lignin, which indicated that the greater part of the decomposition occurred prior to 35 days and then gradually ceased. The final loss in lignin was about two to three times

the magnitude of the apparent increase. This indicates that the loss is not a reversal of the apparent increase after a growth period of several weeks, but represents an actual decomposition of the lignin. Between 25° and 30° C. there was no significant difference in so far as lignin decomposition is concerned.

2. THE EFFECT OF DIFFERENT NEUTRALIZING AGENTS

Effect of Three Different Neutralizing Agents at Two Temperatures and the Same pH

Brucite, ammonium carbonate, and a mixture of calcium carbonate and magnesium carbonate were used as neutralizing agents. Each experiment was carried out at two different temperatures, 25° and 30° C., and each series was analysed at three time intervals, initial time, 10 days, and 20 days. The pH of the different media were adjusted to approximately the same level.

The addition of 5 gm. per litre of calcium carbonate and 8 gm. per litre of magnesium carbonate to the first series and 20.5 gm. freshly calcined brucite to the second series gave pH values of 6.50 and 5.98, respectively. After sterilization and addition of 5 ml. of a sterilized mixture of 1.2% each of ammonium mono- and dihydrogen phosphate to each 100 ml. of the medium the pH values were 5.50 and 5.67. In the third series the ammonium carbonate was sterilized separately and added with the phosphates at the rate of 1.2 gm. per litre. This gave a final pH of 5.71. The results of the analyses are summarized in Table II.

As has been observed in previous experiments, after incubation the control flasks tended to become slightly more acid. The test flasks, on the other

TABLE II

THE EFFECT OF pH AND COMPOSITION OF MEDIA ON GROWTH OF *E. adiposa* ON SULPHITE WASTE LIQUOR MEDIA

Series No.	Growth period, days	Temperature, 25° C.			Temperature, 30° C.		
		pH	Sugar fermented, %	Lignin decomposed, %	pH	Sugar fermented, %	Lignin decomposed, %
1	0	5.50	0.00	0.00	5.80	0.00	0.00
	10	5.91	62.70	2.85	5.54	49.53	2.82
	20	6.88	74.24	8.08	6.77	76.96	8.68
2	0	5.67	0.00	0.00	5.45	0.00	0.00
	10	5.24	64.03	0.51	5.49	47.90	2.01
	20	6.62	72.52	4.17	6.46	80.82	6.50
3	0	5.71	0.00	0.00	5.62	0.00	0.00
	10	5.39	84.55	8.62	4.80	*	*
	20	5.66	89.32	8.78	4.88	*	*

* Cultures did not grow.

hand, became more basic and at 20 days had a pH of approximately 6.90. The more extensive the growth of the fungi, the higher the pH was found to be.

The sugar content at the beginning of the experiment was about 22.0 gm. per litre. This remained fairly constant throughout the course of the experiment in the control flasks. In the inoculated cultures at 10 days over 50% of the sugar had been fermented and at 20 days this had risen to 80%, which appears to be about the limit for *E. adiposa*.

The lignin values at the beginning agree very closely and show a content of about 62 gm. per litre. The lignin content of raw sulphite liquor is very close to this figure, so it can be assumed that the addition of calcium and magnesium carbonate in quantities sufficient to bring the pH up to 5.5 to 6.0 does not cause any precipitation of the lignosulphonate. The loss of lignin is approximately the same at both 25° and 30° C.

In the second series the pH in the control flasks showed a progressive increase in acidity, while the test flasks on the other hand showed a decrease in acidity to an average pH of 6.70. Those with poor growth had a correspondingly lower pH. The rate and extent of lignin decomposition was about 50% less than found in the previous series. While the growth on media neutralized with brucite was quite satisfactory, it was apparent from the variations between the individual controls that this neutralizer was interfering with the lignin determinations. Consequently it was not used for further experimental work.

In the third series growth was very rapid at 25° C. and, as indicated by the amount of sugar fermented, was complete in 10 days. There was relatively little change in pH during the experiment. At 30° C., however, the medium became quite acid (pH 4.80) and no growth occurred.

At 10 days the lignin in the controls had undergone an apparent increase from an initial 62 gm. per litre to 64 gm. per litre. In the inoculated flasks the lignin was still about 6 gm. per litre lower than the corresponding controls. At 20 days there was little further change in the loss of lignin in the test flasks. This seems to indicate that the decomposition of lignin paralleled the fermentation of sugar and when the sugar had been removed the decomposition of lignin almost ceased. In the first series in which the sugar was removed more slowly, the lignin was reduced slowly at first and then more rapidly as the sugar reserve was used up. It may be possible that the organism requires some fermentable sugars as a source of energy while adapting itself to the utilization of lignin. If the sugar is rapidly fermented, it appears to leave the organism unable to continue the lignin fermentation effectively.

A comparison of the different treatments carried out in this experiment shows that the best results have been obtained by using a mixture of calcium and magnesium carbonates as the neutralizing agents. The brucite treatment introduced technical difficulties in the determination of lignin. The ammonium carbonate made the nitrogen content unreliable after sterilization

due to loss of ammonia. The difference in the breakdown of lignin for 25° and 30° C. was not significant enough to warrant further use of the higher temperature. In fact, the organism failed to grow in the third series at the higher temperature.

The Effect of Basic Carbonate Neutralizers

In this experiment *E. adiposa* was grown in liquor that had been neutralized with sodium, calcium, or magnesium carbonate. The pH was adjusted to a different level with each material.

The addition of 3.4 gm. per litre anhydrous sodium carbonate, 6.5 gm. per litre calcium carbonate, and 6 gm. per litre magnesium carbonate before sterilization gave final pH values of approximately 5.5, 5.9, and 6.8, respectively. In all instances 5 ml. of a sterile solution containing 1.2% each of ammonium mono- and dihydrogen phosphate was added to each 100 ml. of media. In this experiment the flasks were incubated at 25° C. only.

The results obtained are reported in Table III. In control flasks neutralized with sodium carbonate the pH dropped to 5.0 and remained fixed there. With calcium carbonate the pH dropped to 5.3 and became stationary. In the test flasks, after the usual initial drop, the pH rose.

TABLE III

THE EFFECT OF DIFFERENT NEUTRALIZING REAGENTS AND pH ON DECOMPOSITION OF SUGARS AND LIGNIN OF SULPHITE WASTE LIQUOR BY *E. adiposa*

Neutralizing agent	Growth period, days	pH	Sugar fermented, %	Lignin decomposed, %
Na ₂ CO ₃	0	5.52	0.00	0.00
	10	5.37	82.30	8.27
	20	5.77	88.52	10.92
CaCO ₃	0	5.90	0.00	0.00
	10	6.12	73.26	4.63
	20	7.02	87.84	8.82
MgCO ₃	0	6.84	0.00	0.00
	10	6.38	60.95	4.64
	20	7.38	83.46	6.58

The fermentation of sugar was most rapid with sodium carbonate. In media treated with both sodium and calcium carbonate the fermentable portion of their sugar was removed in 20 days. Fermentation was considerably slower in the magnesium carbonate media. It appeared that the high initial pH had a retarding effect on growth.

The greatest lignin decomposition occurred in the media neutralized with sodium carbonate; the least in that neutralized with magnesium carbonate.

From Table III it may be seen that the pH of the sodium carbonate is lower than that of the magnesium carbonate media, and so it is possible to

correlate lignin decomposition and pH of the media in this experiment. It is believed that the final pH of the solution is a more important factor than the reagent used to obtain it. While it would be preferable to study the effect of pH alone by using different amounts of the same neutralizing agent, it was found experimentally that only a very narrow pH range could be obtained since sterilization of the media tended to bring them all to the same level. It was therefore necessary to use different neutralizers for each level of pH. For *E. adiposa* the best pH for sugar fermentation and lignin decomposition was around 5.50 to 5.85 but mycelial growth was most rapid at a pH about 6.50.

3. THE EFFECT OF DIFFERENT NITROGEN SOURCES

The nitrogen source is a very important factor in the growth of *E. adiposa* in waste sulphite liquor. Qualitative experiments previously carried out demonstrated that different sources of nitrogen did not give the same amount of growth on agar media. Ammonium nitrogen was one of the best sources, while inorganic nitrate was one of the poorest. A quantitative experiment was set up using six different nitrogen sources.

The culture medium was prepared as previously and neutralized with 5 gm. per litre each of calcium carbonate and of magnesium carbonate. The phosphate source was added before sterilizing as a mixture of 1.2% potassium monohydrogen and 1.2% of dihydrogen phosphate at the rate of 5 cc. of this mixture to each 100 cc. of the medium. The nitrogen sources used were ammonium monohydrogen phosphate, sulphate, carbonate, potassium nitrate, Difco bacto-peptone, and urea. They were added in such quantities that the nitrogen content of the medium would be 4 gm. per litre. In order to keep the phosphate content equivalent in all media, none was added to that containing the ammonium monohydrogen phosphate. The nitrogen solutions were sterilized by filtration through a Jena sintered glass filter. The analyses for pH change, sugar, and lignin content were done at 10 and 20 days. In each set there were two test flasks and one control. The pH of all cultures was approximately 6.00. All were incubated at 25° C. The results obtained are shown in Table IV.

The flasks containing potassium nitrate as a source of nitrogen showed very little growth and the mat was submerged. In all others, growth was fairly rapid, and in about three days the surface of the medium was covered with mats of about equal depth. At the end of 10 days the pH value had undergone little change. At 20 days there was a marked change in pH, most of the cultures having become alkaline with the exception of those containing potassium nitrate and ammonium monohydrogen phosphate. At 20 days, the sugars, except in the potassium nitrate culture, were about 80% utilized; this, as previously observed, is about the maximum fermentation of the sugars of waste sulphite liquor by *E. adiposa*. In the potassium nitrate media, on which growth was poor, about 50% of the utilizable sugar was still left. The general appearance of the other cultures indicated that

TABLE IV

THE EFFECT OF NITROGEN SOURCES ON THE DECOMPOSITION OF SUGARS AND LIGNIN OF SULPHITE WASTE LIQUOR BY *E. adiposa*

Nitrogen source	Initial pH	Analysis at 10 days			Analysis at 20 days		
		pH	Sugar fermented, %	Lignin decomposed, %	pH	Sugar fermented, %	Lignin decomposed, %
(NH ₄) ₂ HPO ₄	6.10	5.89	36.92	3.76	5.86	79.89	0.00
(NH ₄) ₂ SO ₄	6.00	6.40	55.86	5.23	7.45	83.75	9.41
(NH ₄) ₂ CO ₃	6.38	6.56	59.62	6.00	9.11	80.93	7.07
KNO ₃	6.00	5.66	13.42	1.36	5.67	40.11	2.66
Peptone	6.25	6.50	56.28	5.45	7.48	86.10	8.13
Urea	6.10	6.49	48.42	4.53	7.45	82.19	9.07

the active growth period was over as they had turned from white to dark mats which were wrinkled at the edges. The lignin breakdown at 20 days was somewhat greater than at 10 days with the exception of the ammonium monohydrogen phosphate flasks, which after showing a breakdown of 3.76% lignin in 10 days, at 20 days showed no lignin breakdown at all. This appears to be due to a change in metabolism as the pH of this solution never became alkaline as in the others. The precipitate that was formed with β -naphthylamine was not the typical plastic-like substance usually obtained, but was a powdery, reddish material, which weighed more than its control, thus giving the illusionary effect of lignin having been built up again at 20 days. In view of the fact that in the experiments with different neutralizers ammonium monohydrogen phosphate, when used in larger quantities gave excellent growth, rapid sugar fermentation, and lignin breakdown of 8 to 10%, the poor results here may be attributed to the smaller amount used in this experiment. It must be pointed out that this ammonium monohydrogen phosphate medium is not strictly comparable to the others since in the other cultures phosphates were present in the form of potassium salts. The lignin breakdown in the potassium nitrate cultures was negligible. Of the other sources of nitrogen there does not appear to be any preference, all being utilizable and to approximately the same extent. The ammonium sulphate gave the best breakdown, with urea next. Peptone also appears to be a good source of nitrogen.

Less extensive experiments carried out with the amino acid glycine and the amine, asparagine, proved that they are also available sources of nitrogen for this organism.

Discussion

✓ Sulphite waste liquor may be readily converted into a suitable culture medium for the growth of fungi. Since the lignin is present in the form of water soluble calcium lignosulphonate, it is more adaptable for studies on decomposition than insoluble lignin compounds. One of the main disadvantages in using sulphite waste liquor is the fact that the lignin content, as measured by the β -naphthylamine precipitation method, undergoes an

apparent increase over a period of approximately three weeks in sterile culture media. This fact might lead to the conclusion that the loss of lignin may be only a reversal of the apparent increase and that no real loss had occurred. However, it has been shown that the calculated loss is from two to three times the amount of the increase and thus indicates that an actual loss of calcium lignosulphonate has taken place. Further evidence for a real decomposition of the lignin is provided by the relation between growth and lignin breakdown. When growth of the fungus is abundant as with suitable sources of nitrogen, the lignin loss is correspondingly greater than with poor nitrogen sources which give scanty growth. The fact that extensive growth is positively correlated with lignin decomposition indicates that the lignin is being utilized in the process of growth.

~ It will be observed that the lignin breakdown in most cases parallels the sugar fermentation and after the available sugar has been used up, the lignin decomposition tapers off rather quickly. It was thought that it might be possible to stimulate the breakdown of lignin if more sugar were supplied, qualitative tests having shown that available nitrogen and phosphate still remained. Sugar was added in amounts of 1, 3, and 5% to cultures that had been fermenting slowly for six months. Analysis showed they consumed the sugar completely, but there was no further breakdown in the lignin. This would lead to the supposition that only a certain fraction of the lignosulphonate is fermentable and when that part has been consumed the reaction stops. It may be that this conversion yields compounds like humic acid that are very resistant to microbial growth. The nature of the precipitate obtained with β -naphthylamine on these old cultures lends strength to the idea that the lignosulphonate has been changed considerably as it does not give a hard brittle plastic-like precipitate but a powdery grit-like substance. There is evidence to show that in native lignin decomposition it combines with proteins to give substances that are very resistant to bacterial action (6, 13). It is possible that the same type of reaction may occur when fungi are grown on waste sulphite liquor.

! It is important to point out that consistent results in the experiments with different nitrogen sources have been very difficult to obtain. Attempts were made to have the actual amounts of nitrogen in the media identical and this has meant that other factors, probably of equal importance, have been thrown out of their proper proportion. For other reasons yet unexplained, the results have sometimes been poor, for instance, the lignin breakdown with ammonium monohydrogen phosphate in the experiment on nitrogen sources was nil although in previous experiments it has been possible to get a breakdown of 6.7%. Sulphite liquor has such a variety of different substances in its composition that it is difficult to make an identical medium for each experiment and so little is known of the metabolism of *E. adiposa* that slight changes may profoundly influence its growth. Only a further study of the problem will elucidate all the factors governing the growth of the organism in this medium. ✓

Acknowledgments

Grateful acknowledgment for technical assistance is due to Miss M. T. Clement for mycological work and to Mr. A. Castagne for chemical analyses; the authors are also grateful to Dr. F. L. Drayton, Division of Botany, Central Experimental Farm, Ottawa, for providing the culture of *Endoconidiophora adiposa*.

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BIOLOGICAL DECOMPOSITION OF CHEMICAL LIGNIN

II. STUDIES ON THE DECOMPOSITION OF CALCIUM LIGNOSULPHONATE BY WOOD DESTROYING AND SOIL FUNGI¹

BY G. A. LEDINGHAM² AND G. A. ADAMS³

Abstract

The growth and lignin decomposing properties of 106 cultures of wood destroying and soil fungi have been studied on a synthetic calcium lignosulphonate medium. Certain species of *Fusarium* and *Alternaria*, decomposing a maximum of 12 and 18% lignin, respectively, were the most effective in utilizing the lignosulphonate. Although a few species of wood destroyers were equally effective, in general this group of fungi showed great variation and was more difficult to cultivate on the medium employed. A slight positive correlation was found between the Bavendamm tannic acid reaction for identifying lignin decomposing fungi and the lignosulphonate breakdown after 60 days' growth.

In a previous paper (1) it was demonstrated that the wood staining fungus *Endoconidiophora adiposa* (Butler) Davidson was capable of decomposing approximately 10% of the calcium lignosulphonate of sulphite waste liquor, provided the liquor had been rendered non-toxic and was supplemented with certain essential nutrients. However, it was found that the complex character of sulphite waste liquor presented several problems. The lignosulphonate content of the control media did not remain constant but steadily increased over a period of about 30 days. This made dependable appraisal of the results difficult, as the increase was not always constant over several identical controls. Furthermore, the uniform removal of sulphur dioxide from waste liquor to give a non-toxic medium for growth of many different organisms could not always be accomplished satisfactorily. It was thought that these difficulties could be largely eliminated by the use of a medium containing pure lignosulphonate salts isolated from sulphite waste liquor. After preliminary studies had been carried out in order to develop a satisfactory standard medium, a survey was made of different species or strains of fungi in order to determine their lignin decomposing properties.

Methods

Preparation of Calcium Lignosulphonate

Two litres of sulphite waste liquor were evaporated on a hot plate to a volume of 600 ml. and vigorously aerated at the same time. The solution was filtered when cool, and 12 ml. of concentrated sulphuric acid was added with stirring. A heavy white precipitate of calcium sulphate came down, was filtered off, and washed with water. The pH of the filtrate was adjusted to

¹ Manuscript received September 5, 1941.

Contribution from the Division of Biology and Agriculture, National Research Laboratories, Ottawa. Issued as N.R.C. No. 1023.

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approximately 5.00 with calcium carbonate (20 gm.). This pH adjustment was necessary as the strongly acid solution rendered the lignosulphonate soluble in the subsequent treatment with alcohol. The solution was then centrifuged to remove the calcium sulphate. Two hundred and fifty ml. of the solution was then added to two litres of 95% ethanol at the rate of about two drops per second, the alcohol solution being mechanically stirred at a rapid rate at the same time. The calcium lignosulphonate precipitated out in the alcohol solution, but care had to be taken to stop the addition of the liquor before the precipitate became brown and sticky. After pouring the alcohol off, the precipitate was repeatedly washed with 95% ethanol, then taken up in 125 ml. of water and again precipitated from two litres of 95% ethanol. This second precipitate was washed with fresh 95% ethanol and separated on a Buchner filter. The precipitate was then dried in a warm draught of air. When dried and ground, it was a light buff coloured powder, which was stable when stored out of contact with air. The yield was about 60 gm. from two litres of liquor.

✓ The Isolation and Selection of Lignin Decomposing Fungi

(Fungi are generally regarded as being among the most active of the micro-organisms that break down the native lignin in plant materials in soils and composts.) Waksman and Hutchings (7) found that species of *Fusarium* and *Alternaria* could cause decomposition of phenol lignin and Sadasivan (5) has shown that *Fusarium culmorum* (W. G. Smith) Sacc. is an important primary colonizer of wheat straw buried in soil. Cultures for this study were isolated by inoculating sulphite waste liquor media with bits of rotting wood, leaf mould, heating manure, well rotted manure, mouldy hay, straw and cereal grains, rotting fruit and vegetables, and soil. Certain species survived and flourished in this medium and pure cultures of these were isolated. In addition, cultures of wood destroying fungi were obtained from the Division of Botany, Central Experimental Farm, Ottawa, and the Forest Products Laboratory, Washington, D.C. Several species of *Fusarium* were obtained from the Dominion Laboratory of Plant Pathology at Saskatoon, Sask., and strains of *Alternaria*, *Phoma*, *Melanospora*, and a few other genera, many of which undoubtedly occur in soil, were obtained from a collection isolated from seeds in the Division of Botany, Central Experimental Farm, Ottawa.

Forest mycologists have attempted to classify wood destroying fungi into cellulose and lignin decomposing types by growing them on media containing tannic acid. According to Bavendamm (2) who devised the test, those species that produce a brown discoloration on this medium elaborate enzymes that are capable of breaking down lignin. Davidson, Campbell, and Blaisdell (3) tested the reactions of 210 wood destroying fungi on gallic and tannic acid media and found that 80% of the negative reactors were correctly diagnosed as brown rot fungi and 96% of the positive reactors were diagnosed as white rot fungi. Since white rot fungi attack the lignin more actively than the cellulose, it appeared that this test might be useful in selecting lignin decomposing fungi for this work. It also appeared worth while to determine

whether there was any correlation between the reaction on tannic acid media and actual breakdown of lignosulphonate acids. ✓

Preparation of the Culture Media

The same medium was used for the growth of all the species tested. Obviously a standard medium could not be expected to be entirely satisfactory for all the different fungi tested as their nutritional requirements are not identical. However, in order to test a large number of species, it was not practical to attempt nutritional studies with each to determine the optimum conditions for growth. The standard medium that was used for this general survey was, however, very satisfactory except for a few species. It was made up as follows:

Ammonium monohydrogen phosphate	5.0 gm.
Potassium chloride	1.0 gm.
Magnesium sulphate	1.0 gm.
Ferrous sulphate	0.02 gm.
Dextrose	20.0 gm.
Calcium lignosulphonate	50.0 gm.
Distilled water	1000 ml.

The calcium lignosulphonate was added in approximately the same amount as is present in sulphite waste liquor. It should be noted that while 5 gm. per litre of ammonium monohydrogen phosphate would be high in an ordinary medium, there is a heavy precipitate of phosphate produced when this is added to calcium lignosulphonate, hence the amount in solution at any one time is considerably less than 0.5%. It was determined experimentally that this amount gave better growth with most species than a smaller quantity and no detrimental effects were noted even when 10 gm. per litre was added. This culture medium also contains glucose. While it would have been preferable to have had lignosulphonate as the only carbon source, previous studies indicated that the growth without additional carbon was so meagre that little change occurred in the medium. With a readily available carbon source present growth of the fungus was luxuriant, and with those species capable of attacking the lignosulphonate there was a slow but gradual breakdown so long as growth of the fungus occurred.

In preparing the medium, the calcium lignosulphonate and magnesium sulphate were dissolved in half the volume of boiling water and the ammonium monohydrogen phosphate, potassium chloride, and ferrous sulphate in the remainder. Fifty ml. of each solution was then pipetted into a 500-ml. flask. This assured an even distribution of the precipitate that occurred when ammonium monohydrogen phosphate, magnesium sulphate, and calcium lignosulphonate interacted. The medium was then sterilized at 120° C. for 20 min. During sterilization the pH dropped from approximately 7.00 to 5.50.

After cooling, the flasks to be inoculated received 1 ml. of a spore suspension of the organisms being tested. Uninoculated control cultures were incubated with the inoculated. Usually two cultures, with a control, were grown and

analysed, one at each of two dates. However, in a few tests designed to test the accuracy of the method, triplicate cultures of certain species were analysed. Analyses were made at intervals of 10 and 20 days for most of the cultures except the wood destroyers, which, because of slow growth, were done at 30- and 60-day intervals. Whenever an analysis was made, three uninoculated flasks of medium were analysed to give a base line for lignin.) The methods of analysis used were the same as those described in the previous paper in this series (1).

Experimental Results

THE DECOMPOSITION OF CALCIUM LIGNOSULPHONATE BY SOIL FUNGI

Species of Alternaria

Thirty-three isolates of *Alternaria* were tested on the calcium lignosulphonate medium. The analytical results which include changes in the pH of the media and percentage loss of the sugar and lignin at 20- and 30-day intervals are shown in Table I, in decreasing order of lignin loss after 20 days' incubation. The initial pH of the medium was 4.70 and in all cultures the pH had decreased at 10 days. After 20 days' growth it was found that in some instances the pH was higher than its corresponding value at 10 days, while in others it still continued to fall. These pH values are undoubtedly associated with the utilization of the sugar. At 10 days there was a correlation coefficient of $- .578$ between the percentage of sugar fermented and the pH of the solution, which indicates that the most active fermentation produced the greatest acidity. At 20 days the correlation between pH and sugar was $.386$, indicating that the organisms that had utilized the most sugar had now started utilizing the acids. On the other hand, the percentage of lignin decomposed showed no demonstrable association with either pH or fermentation of sugar at either 10 or 20 days. There was no consistent trend for either pH or lignin, consequently one could not predict at 10 days what the results would be at 20. With sugar there was, however, a significant correlation, though small for purposes of prediction ($r = .51$) between percentage of sugar lost at 10 and 20 days. The 33 cultures of *Alternaria* had marked differences in their ability to decompose lignin, showing losses of 3 to 18% of the lignosulphonate present in the solution.

Species of Fusarium

The analytical results obtained from the growth of 13 different species of *Fusarium* on calcium lignosulphonate media are shown in Table II. The medium and experimental conditions were similar to those used with the *Alternaria* species but the growth period was extended to 40 days in order to find out whether a longer fermentation period would influence lignin breakdown.

It is apparent from this table that the sugar fermentation was practically complete at 20 days. In 6 out of the 13 species studied there was an increase in the lignin decomposition at 40 days, as compared with 20 days, but the remainder showed an apparent decrease. This effect was frequently observed

TABLE I
ANALYTICAL RESULTS FROM *Alternaria* CULTURES GROWN ON CALCIUM
LIGNOSULPHONATE MEDIA

N.R.C. culture No.	Incubation time, days					
	10			20		
	pH	Sugar fermented, %	Lignin decomposed, %	pH	Sugar fermented, %	Lignin decomposed, %
T-G-2	4.39	42.2	8.1	4.62	98.4	18.2
L-21	3.78	25.1	5.1	3.35	77.9	16.9
D-96	4.05	23.7	4.1	4.01	49.3	16.8
T-S-29	4.05	81.6	15.1	5.43	98.1	16.0
L-119	3.60	84.5	2.4	5.50	97.6	15.3
L-13	3.80	32.6	7.6	3.87	46.2	15.0
T-G-36	3.55	63.4	3.7	3.85	98.4	15.0
P-1	3.60	21.7	6.6	3.45	89.1	14.3
T-G-26	4.59	7.8	2.6	3.70	76.8	13.6
T-G-10	4.60	15.4	7.2	3.95	93.0	13.2
L-52	4.02	17.0	1.4	3.59	57.6	11.8
T-G-1	4.65	32.2	8.8	4.62	97.0	11.6
T-G-27	4.42	21.1	5.2	4.12	95.1	11.5
T-G-23	4.58	17.1	9.7	3.72	95.2	11.1
L-113	3.72	45.6	3.8	3.75	98.0	10.5
T-G-29	4.28	27.4	5.1	4.00	97.5	10.3
L-155	3.50	71.8	1.8	3.89	97.4	10.2
T-G-38	4.18	34.7	4.8	3.75	74.9	9.9
T-G-33	4.43	27.1	5.2	3.73	51.7	9.3
T-G-19	4.30	28.6	4.0	5.02	97.8	9.2
E-O-2	3.72	73.6	1.2	4.35	98.7	8.7
T-G-37	4.32	22.4	7.0	3.40	94.8	8.3
T-G-22	4.62	25.8	7.8	3.78	95.1	8.0
T-G-35	3.50	74.5	8.2	4.60	97.6	7.7
L-86	3.72	12.3	+1.4	3.81	38.4	7.7
T-G-3	4.60	35.2	8.2	4.22	98.4	7.5
T-G-18	4.65	2.0	0.3	4.09	33.0	6.7
T-G-28	4.35	29.1	4.1	4.30	97.5	6.3
T-G-34	3.75	59.2	6.0	4.10	97.6	5.1
T-G-9	4.42	30.7	5.2	4.10	76.6	5.0
T-G-4	4.35	63.6	3.3	4.89	98.4	4.5
T-G-25	4.35	32.5	3.3	5.05	97.4	3.0
T-G-8	4.42	34.9	1.5	4.89	98.7	2.8

in cultures that had been growing for a long period of time. The lignin precipitate as obtained by β -naphthylamine had been changed and became a reddish powder instead of a brittle black plastic. This precipitate weighed more than the corresponding one at 20 days and gave the apparent effect of lignin having been built up during the last 20 days. This may have been due either to products of metabolism precipitating and being calculated as lignin, or to an influence on the precipitation of calcium lignosulphonate so that it was more complete (normally β -naphthylamine precipitates only 68% of calcium lignosulphonate present).

At 20 days there was a correlation coefficient of .67 between pH and sugar values, and between lignin and pH the value was .53. This means that low pH values were associated with almost complete sugar fermentation

TABLE II
ANALYTICAL RESULTS FROM *Fusarium* CULTURES GROWN ON CALCIUM
LIGNOSULPHONATE MEDIA

N.R.C. culture No.	Species	Incubation time, days					
		20			40		
		pH	Sugar fer- mented, %	Lignin decom- posed, %	pH	Sugar fer- mented, %	Lignin decom- posed, %
T-S-24	<i>F. culmorum</i>	2.92	83.9	13.4	4.25	90.0	12.5
T-S-23	<i>F. coeruleum</i>	3.40	90.2	8.9	3.60	93.1	11.8
T-S-17	<i>F. orthocera</i>	4.61	93.1	5.2	3.89	92.9	10.9
T-S-20	<i>F. concolor</i>	4.15	94.2	4.8	3.49	94.1	10.7
T-S-38	<i>F. sp.</i>	3.80	93.6	4.9	4.49	94.8	9.5
T-S-35	<i>F. culmorum</i>	4.40	93.5	4.1	4.02	93.5	8.4
T-S-16	<i>F. Equiseti</i>	3.63	93.2	8.3	5.25	91.5	7.3
T-S-26	<i>F. avenaceum</i>	3.65	92.5	6.9	4.67	93.6	7.2
T-S-18	<i>F. solani</i>	3.70	92.3	8.2	5.10	95.1	6.1
T-S-22	<i>F. oxysporum</i>	3.80	92.5	6.1	4.92	91.8	5.9
T-S-19	<i>F. oxysporum</i>	3.52	92.3	9.0	5.41	92.4	5.6
D-163	<i>E. sp.</i>	3.40	93.6	8.2	4.55	92.9	4.0
T-S-39	<i>E. sp.</i>	4.88	94.8	4.4	5.15	94.7	1.4

and relatively high lignin breakdown. At 40 days, however, the correlation between sugar and pH was not significant ($r = .07$). The correlation between lignin and pH was $-.78$, which indicated that now the high pH was associated with a low lignin breakdown. The partial correlation coefficient of $-.79$ shows that this relation was independent of the effect of sugar. The *Fusarium* cultures all brought about rapid fermentation of the sugar but they did not carry the lignin decomposition as far as certain *Alternaria* cultures. There was no correlation between the 20- and 40-day periods for any of the factors studied.

Miscellaneous Soil Fungi

A number of fungi belonging to different genera that may be found on decaying vegetable matter, or in soil, were tested on the calcium lignosulphonate media. They have been arranged in Table III in the order of those giving the greatest lignin breakdown at 20 days.

Since this is a heterogeneous group of organisms there are wide variations in the final pH of the culture medium and also in the amount of sugar fermented. The *Botrytis* and *Cephalothecium* species were both active in the fermentation of sugar and at the same time produced a low pH. The two cultures of *Aspergillus* fermented the sugar very completely in 10 days, but did not alter the pH greatly. As might be expected from an unrelated series of organisms, the coefficients of correlation between pH and sugar, pH and lignin, and lignin and sugar, did not reach significance except in the case of lignin decomposition and pH at 20 days, when there was some tendency for increased decomposition to be associated with high pH. However, the corre-

TABLE III

ANALYTICAL RESULTS FROM MISCELLANEOUS SOIL FUNGI GROWN ON CALCIUM LIGNOSULPHONATE MEDIA

N.R.C. culture No.	Name	Incubation time, days					
		10			20		
		pH	Sugar fer- mented, %	Lignin decom- posed, %	pH	Sugar fer- mented, %	Lignin decom- posed, %
T-G-7	<i>Phoma</i> sp.	4.25	48.4	5.2	4.91	97.6	12.3
C-66	<i>Trichoderma</i> sp.	4.20	71.0	9.3	4.30	97.9	10.1
T-G-72	<i>Epicoccum purpurascens</i>	4.10	14.6	4.2	3.50	63.8	9.8
L-125	<i>Trichoderma</i> sp.	6.55	73.8	1.4	7.18	95.6	9.7
D-61	<i>Aspergillus</i> sp.	5.88	95.5	3.7	5.81	96.3	8.4
T-G-63	<i>Stemphylium</i> sp.	3.93	45.7	4.1	3.75	56.1	8.4
T-G-16	<i>Stemphylium</i> sp.	4.28	34.6	4.8	4.38	96.9	8.0
T-G-51	<i>Phoma vulgaris</i>	3.99	45.2	2.1	3.80	65.6	7.0
T-G-86	<i>Botrytis</i> sp.	2.92	82.3	6.1	5.39	96.2	6.4
T-G-54	<i>Phoma</i> sp.	4.41	27.7	5.8	3.85	62.0	6.3
T-G-79	<i>Cephalothecium</i> sp.	2.80	95.1	6.8	3.48	98.1	6.1
T-G-65	<i>Stemphylium</i> sp.	4.11	11.3	2.8	3.15	84.7	6.0
L-37	<i>Aspergillus niger</i>	4.58	93.6	1.9	5.00	100.0	5.7
T-G-17	<i>Stemphylium</i> sp.	4.00	80.2	1.8	5.10	99.4	5.7
T-G-84	<i>Cladosporium</i> sp.	3.95	20.8	1.7	3.53	42.6	5.7
T-G-91	<i>Stemphylium botryosum</i>	3.48	78.9	4.2	3.39	91.1	5.6
T-G-48	<i>Melanospora</i> sp.	4.01	41.2	2.7	3.68	71.3	5.3
T-G-66	<i>Stemphylium</i> sp.	3.69	59.5	2.3	3.25	95.0	5.3
D-92	<i>Botrytis</i> sp.	3.65	2.7	0.7	4.30	30.3	5.2
D-2	<i>Trichoderma</i> sp.	4.30	37.5	3.0	3.90	100.0	4.3
T-G-87	<i>Cephalothecium</i> sp.	2.85	91.6	6.2	3.22	97.5	3.6
T-G-53	<i>Phoma melina</i>	4.25	19.9	3.4	4.05	38.8	3.1
H-2	<i>Mucor racemosus</i>	4.27	75.4	0.7	5.4	100.0	2.8
T-G-75	<i>Botrytis</i> sp.	2.95	88.0	2.5	4.40	96.2	2.8
T-G-73	<i>Verticillium</i> sp.	4.25	13.2	1.4	4.18	20.4	1.8
T-G-62	<i>Chaetomium globosum</i>	4.32	29.1	1.9	4.03	51.5	0.3

lation between the results at 10 and 20 days for these different factors was significant, being 0.48 for pH, 0.77 for sugar, and 0.44 for lignin. This means that given the analysis at 10 days it is possible to some extent to predict the trend at 20 days, particularly in the case of sugar. In this group of 26 different isolates from 13 different genera, the majority of the species used were not particularly promising as lignin decomposers.

THE DECOMPOSITION OF CALCIUM LIGNOSULPHONATE BY WOOD DESTROYING FUNGI

In addition to common soil fungi, and species abundant on decaying vegetation that might hold promise for lignin breakdown, there remained the large group of fungi that attack and bring about various rots in wood. Thirty different species belonging to several genera were used to inoculate calcium lignosulphonate solutions of the same composition as previously used. Since these fungi generally grow much more slowly than the common moulds on

synthetic media, it was necessary to extend the growth period considerably, and analyses were made at 30- and 60-day intervals. The results are shown in Table IV.

TABLE IV
ANALYTICAL RESULTS FROM WOOD DESTROYING FUNGI GROWN ON CALCIUM
LIGNOSULPHONATE MEDIA

Division of Botany C.E.F. culture No.	Species	30 days			60 days		
		pH	Sugar fermented, %	Lignin decomposed, %	pH	Sugar fermented, %	Lignin decomposed, %
8461	<i>Poria ferrea</i>	4.52	24.3	12.1	3.25	47.9	14.8
6893	<i>Polyporus abietinus</i>	4.12	37.4	14.2	4.10	63.7	13.6
8445	<i>Fomes robustus</i> var. <i>tsugina</i>	4.50	24.9	11.8	4.40	30.5	12.7
3445	<i>Polyporus anceps</i>	4.30	41.7	12.6	3.89	94.7	11.9
598	<i>Poria subacida</i>	3.95	31.4	11.0	3.95	42.6	11.9
8217	<i>Trametes tenuis</i>	4.32	23.7	12.5	3.95	33.0	11.7
7997	<i>Polyporus tuberaster</i>	3.90	37.0	9.8	3.73	71.4	10.7
7526	<i>Polyporus compactus</i>	4.32	31.6	8.6	4.05	54.7	10.5
8549	<i>Polyporus resinosus</i>	3.78	53.1	12.1	3.75	76.3	10.7
7403	<i>Poria obliqua</i> (Sterile <i>Fomes igniarius</i>)	4.23	34.1	6.9	3.88	59.3	9.0
8754	<i>Poria Weirii</i>	4.05	25.1	8.4	3.89	31.0	7.6
8049	<i>Polyporus tulipiferus</i>	3.95	56.5	7.8	3.89	81.3	6.2
9214	<i>Fomes fomentarius</i>	3.25	52.5	9.7	3.21	55.1	6.1
3580	<i>Fomes Everhartii</i>	4.33	41.5	4.7	3.95	86.6	5.6
9235	<i>Polyporus ochroleucus</i>	4.15	45.5	10.6	3.89	76.0	5.6
8415	<i>Fomes annosus</i>	3.85	47.9	11.0	3.95	85.6	5.1
5885	<i>Polyporus pargamensis</i>	4.20	27.3	-4.1	4.00	51.9	4.9
7531	<i>Polyporus pubescens</i>	3.25	79.1	9.2	5.35	95.1	1.1
7120	<i>Fomes pinicola</i>	1.82	35.5	-4.0	1.97	34.4	-0.1
	<i>Lenzites trabea</i>	3.45	86.3	12.7	3.95	94.1	-0.7
2163	<i>Fomes Pini</i>	4.28	32.9	-2.9	3.92	69.2	-0.7
8214	<i>Fomes officinalis</i>	1.85	29.2	0.0	1.71	41.3	-1.0
8448	<i>Poria ambigua</i>	4.43	95.6	3.2	5.00	97.3	-1.2
7340	<i>Poria ferruginea-fusca</i>	3.62	95.6	0.8	3.70	95.2	-3.3
8282	<i>Poria</i> sp.	1.75	56.6	-3.5	1.68	54.8	-3.4
8787	<i>Merulius lacrymans</i>	3.39	24.4	-3.2	2.13	58.7	-3.4
8240	<i>Polyporus oregonensis</i>	3.69	98.2	-1.5	3.81	84.5	-3.6
8008	<i>Fomes lobatus</i>	3.81	55.5	2.1	5.00	94.5	-3.8
8183	<i>Polyporus versicolor</i>	3.85	79.1	13.8	4.40	95.1	-4.2
8287	<i>Polyporus dichrous</i>	4.49	2.97	-4.7	4.60	17.5	-6.9

The most outstanding difference in the effects brought about by wood destroying fungi as compared with soil fungi on calcium lignosulphonate media is the tendency for many of them to bring about an apparent increase of the amount of lignin in the solution. This was accentuated by a long growth period, but even at 30 days the effect was noticeable. Owing to the slow and scanty growth it is doubtful whether analyses at an earlier period would have yielded satisfactory information. It is worth noting that three cultures, *Fomes pinicola* (Swartz) Cooke, *Fomes officinalis* (Vill.) Fries, and *Poria* sp. gave very low pH values at both 30 and 60 days, indicating strong acid formation. With the exception of *Poria ambigua* Bres., *Poria ferruginea-fusca*

Karst., and *Polyporus oregonensis* (Murr.) Kauf. which utilized over 95% of the sugars in 30 days, these fungi were not very active sugar fermenters. Of the 30 species, about one-third decomposed over 10% of the lignin at the end of 60 days, and this was in most cases equally marked at 30 days, as indicated by the correlation $r = .69$ between the amount of lignin decomposed at 30 and 60 days. There was also a significant positive correlation for pH and for sugar between the 30- and 60-day periods. At 30 days there was a moderate but significant correlation between lignin breakdown and pH ($r = .39$). This relation no longer existed at the 60-day period ($r = .11$). At 60 days the greater the amount of sugar fermented the higher the pH became on the average as indicated by the correlation factor (.37).

Except when there was a high lignin breakdown, the precipitate of calcium lignosulphonate with β -naphthylamine was of the type described for the *Fusarium* cultures, which showed an apparent increase in lignin content after a 40-day growth period. By means of spectroscopic analysis it has been possible to study this effect in more detail and offer an explanation for this apparent increase in lignin. These observations will be presented in a later paper.

REPRODUCIBILITY OF THE LIGNIN DECOMPOSITION RESULTS

Since the previous experiments were all done on a single culture of each of the organisms, it was thought advisable to repeat the growth of certain cultures in triplicate in order to confirm their lignin decomposing action. It has been found in previous work on fungous cultures that satisfactory repetition of results is often difficult to obtain. Consequently five cultures of *Alternaria* and two of *Fusarium* which gave the best decomposition of calcium lignosulphonate in the general experiments were repeated in triplicate. The growth period was prolonged to 40 days and the analyses carried out at 20 and 40 days. The 20-day analyses provided a comparison with the previous single growth experiment, and the 40-day period allowed further changes to be measured. The controls were in triplicate. The results are shown in Table V.

It will be seen from the comparisons in Table V that with the exception of *F. avenaceum* (Fr.) Sacc. and *Alternaria* sp. (D-96) the general growth as measured by the sugar removal was slower in the triplicates than in the single experiment. This effect is reflected in the lignin breakdown since it is correspondingly lower at the end of 20 days in the triplicates. However, at the end of 40 days' growth the triplicates and the single cultures compare very favourably. The agreement between the individual replicates in the experiment are satisfactory both for sugar and lignin loss. In *Alternaria* sp. (L-21) flasks that had poor growth and relatively lower sugar fermentation at 20 days, had a correspondingly low lignin breakdown, and although the fermentation of sugar was complete at 40 days, the lignin breakdown was not as great as in those cultures with better sugar utilization. In general, the mean of the triplicates compares favourably with the result of the single experiments. The variation between individual uninoculated controls was less than 0.5%.

TABLE V

COMPARISON OF SINGLE AND TRIPPLICATE ANALYSIS OF *Alternaria* AND *Fusarium* CULTURES

N.R.C. culture No.	Species	Original analysis, 20 days			Triplicate analysis, 20 days			Triplicate analysis, 40 days		
		pH	Sugar fer- mented, %	Lignin decom- posed, %	pH	Sugar fer- mented, %	Lignin decom- posed, %	pH	Sugar fer- mented, %	Lignin decom- posed, %
D-96	<i>Alternaria</i> sp.	4.01	49.33	16.80	3.65	49.4	9.9	3.13	97.5	16.6
					3.63	45.9	10.6	3.11	94.0	18.3
					3.58	50.1	11.9	3.17	86.6	15.8
L-13	<i>Alternaria</i> sp.	3.87	46.24	15.06	4.00	13.2	3.6	2.60	89.0	12.8
					3.98	13.9	1.8	2.72	81.9	13.1
					4.00	7.5	3.3	2.83	73.2	13.4
L-21	<i>Alternaria</i> sp.	3.35	77.90	16.98	3.57	42.8	9.1	3.01	97.5	13.0
					3.50	46.3	8.4	2.99	94.0	12.7
					3.70	31.6	5.2	3.98	93.3	8.3
T-G-26	<i>Alternaria</i> sp.	3.70	76.81	13.62	3.78	45.1	7.2	3.25	83.6	13.1
					3.75	42.8	6.8	3.38	86.9	8.9
					3.69	53.8	6.2	3.30	95.1	12.3
T-S-29	<i>Alternaria</i> sp.	3.78	86.76	13.84	3.71	45.7	7.9	3.05	96.9	9.2
					3.79	45.7	7.8	3.01	97.5	11.0
					3.70	45.7	7.3	3.00	96.3	11.4
T-S-26	<i>Fusarium avenaceum</i>	3.65	92.50	6.93	3.55	97.4	9.5	3.10	95.7	11.7
					3.55	97.4	8.6	3.05	95.6	10.3
					3.53	97.4	11.6	3.07	96.9	10.3
T-S-24	<i>Fusarium culmorum</i>	5.53	97.59	10.26	2.92	83.9	13.4	4.25	90.0	12.5
					3.10	87.2	13.1	3.95	90.6	10.2
					3.20	86.0	14.0	3.50	89.4	10.5

The above experiment shows that the agreement between individual cultures inoculated and grown for the same period and all analysed together is satisfactory. This, however, does not explain differences that arise when cultures identical in preparation and treatment are grown at different periods. In the above experiment the greatest differences are not between replicate cultures cultivated simultaneously but between replicate cultures inoculated and grown at different time intervals. In order to obtain more precise information on this point, cultures of each of the five organisms used in the previous experiment were grown without replication. Three days later an identical experiment was set up and inoculated. Both were analysed at the end of 20 and 30 days' growth. A statistical analysis of the chemical results was made. The results are shown in Table VI, and an analysis of variance is shown in Table VII.

It is apparent from Table VII that there was a satisfactory agreement for the pH and sugar determinations from replicate trials at different times. For the lignin determination, however, the major portion of the observed

TABLE VI
REPRODUCIBILITY OF RESULTS FROM TWO SEPARATE INOCULATIONS.

N.R.C. culture No.	Species	Analyses at 20 days						Analyses at 30 days					
		pH		Sugar gm./litre		Lignin gm./litre		pH		Sugar gm./litre		Lignin gm./litre	
		Inoculation						Inoculation					
		1	2	1	2	1	2	1	2	1	2	1	2
T-G-26	<i>Alternaria</i> sp.	4.12	4.08	8.5	2.0	24.9	22.1	3.83	5.52	1.2	0.4	22.6	22.9
T-S-24	<i>Fusarium cul- morum</i>	5.40	5.33	0.8	0.5	25.3	22.7	5.45	5.50	0.7	0.4	24.6	24.0
T-S-26	<i>Fusarium avenaceum</i>	3.75	4.03	0.7	0.7	24.8	22.5	4.60	4.10	0.4	0.9	23.3	22.2
L-13	<i>Alternaria</i> sp.	3.98	3.98	11.7	10.0	25.7	21.7	4.38	3.38	1.4	1.1	22.9	22.1
D-96	<i>Alternaria</i> sp.	3.85	3.87	2.3	2.4	24.7	20.9	5.22	5.30	1.4	1.5	23.4	22.8
	Control	4.65	4.65	22.1	23.4	27.8	24.6	4.65	4.71	19.6	23.2	26.9	25.7

TABLE VII
ANALYSIS OF VARIANCE OF RESULTS FROM SEPARATE INOCULATIONS

Source of variance	pH		Sugar ¹		Lignin	
	D.f.	Mean square	D.f.	Mean square	D.f.	Mean square
Replications	1	0.0063	1	4.0861	1	21.4326**
Organisms	5	1.0864*	4	20.2154**	5	6.6352**
Organisms × time	5	0.3009	4	16.8855**	5	0.4794
Time of analysis 20 or 30 days	1	1.0209	1	46.1472**	1	0.8067
Error	10	0.2096	9	2.1116	11	0.9753

* Exceeds mean square error, 5% level of significance.

** Exceeds mean square error, 1% level of significance.

¹ Degrees of freedom reduced because control sugars not included.

variability occurred between the replicates made at different times and was attributable to factors beyond experimental control. The differences between organisms were consistent across all three criteria and there were also further differences shown by the sugar analysis in relation to time (20- and 30-day periods). The large variability between replicates shown in Table VII is explained by the consistently lower lignin content found in Replicate 2 for all organisms as compared with Replicate 1 at 20 days. The effect is not so pronounced at 30 days. The results for sugar fermentation show satisfactory agreement and demonstrate significant differences in the ability of the organisms to ferment sugar in 20 days. It was concluded from these experiments that the cultures could be satisfactorily reproduced at different times, but that extreme care is required to avoid bias in the chemical determination of lignin carried out on different occasions.

TANNIC ACID REACTION IN RELATION TO LIGNIN DECOMPOSITION

Since analytical results on the breakdown of chemical lignin by a number of wood destroying fungi were available, it was decided to culture these species on tannic acid media and determine the correlation between the reaction and the amount of lignin decomposed. A medium containing 1.5% malt extract, 0.5% tannic acid, and 2.0% agar was used. The malt extract and agar were dissolved in 850 ml. of water, the tannic acid in 150 ml. and sterilized separately. The two solutions were mixed just before pouring the plates. The cultures were incubated for six days and the diameter of the colony and halo produced was measured. The different cultures were classified into six groups according to their reaction. These are as follows:

Class O: Negative, no colour change under or around inoculum.

Class I: No colour change under or around the fungous mat.

Class II: Diffusion zone, light to dark brown, under inoculum, visible from under side.

Class III: Diffusion zone light to dark brown under most of mat but not extending beyond the edge (halo, 1 to 5 mm.).

Class IV: Diffusion zone light to dark brown, extending a short distance beyond the edge of the mat or inoculum (halo, 6 to 10 mm.)

Class V: Diffusion zone very dark brown, extending considerably beyond the edge of mat or inoculum (halo, over 10 mm.).

The relation between this tannic acid reaction and calcium lignosulphonate decomposition found after 30- and 60-day growth periods is shown in Table VIII. The species have been arranged in three groups according to the intensity of reaction produced on the tannic acid medium.

The data in Table VIII were analysed statistically and it was found that between groups there was a slight but significant correlation between lignin breakdown and tannic acid reaction at 60 days but not at 30 days. If an organism is selected that belongs to Class IV or V with respect to tannic acid reaction, it may be predicted that its lignosulphonate decomposing ability will probably be better than if it were selected from the other classes. However, in view of the variation in lignin decomposition shown by species giving the same tannic acid reaction, the practical value of such a test would seem to be doubtful.

Most of the *Alternaria* and *Fusarium* cultures were also grown on tannic acid media and the reaction studied. Of the *Alternaria* cultures shown in Table I, one culture (D-96) fell into Class I, three cultures belonged to Class II, and the remainder were in Class IV or V. D-96 was one of the best lignosulphonate decomposing species tested. *Fusarium culmorum* and *F. Equisitii* (Corda) Sacc. gave a Class IV reaction and *F. solani* (Martius p.p.) Appel & Wollenw. and *F. oxysporum* Schlecht. a negative effect. There did not appear to be any correlation between this reaction and lignosulphonate breakdown.

TABLE VIII

TANNIC ACID REACTION AND CALCIUM LIGNOSULPHONATE DECOMPOSITION BY
WOOD DESTROYING FUNGI

Species showing reaction of Classes IV and V	Lignin decomposed, %		Species showing reaction of Classes III and II	Lignin decomposed, %		Species showing reaction of Classes O and I	Lignin decomposed, %	
	30 days	60 days		30 days	60 days		30 days	60 days
<i>Poria ferrea</i>	12.1	14.8	<i>Poria subacida</i>	11.0	11.9	<i>Polyporus tulipiferus</i>	7.8	6.2
<i>Polyporus abietinus</i>	14.2	13.6	<i>Trametes tenuis</i>	12.5	11.7	<i>Fomes Everhartii</i>	4.7	5.6
<i>Fomes robustus</i> var. <i>isugina</i>	11.8	12.7	<i>Polyporus compactus</i>	8.6	10.5	<i>Fomes pinicola</i>	-4.0	-0.1
<i>Polyporus anceps</i>	12.6	11.9	<i>Polyporus resinusus</i>	12.1	10.7	<i>Poria</i> sp.	-3.5	-3.4
<i>Polyporus tuberaster</i>	9.8	10.7	<i>Poria Weirii</i>	8.4	7.6	<i>Merulius lacrymans</i>	-3.2	-3.4
<i>Poria obliqua</i> (Sterile <i>Fomes igniarius</i>)	6.9	9.0	<i>Fomes Pini</i>	-2.9	-0.7	<i>Polyporus dichrous</i>	-4.7	6.9
<i>Fomes fomentarius</i>	9.7	6.1	<i>Poria ambigua</i>	3.2	-1.2			
<i>Polyporus ochroleucus</i>	10.6	5.6	<i>Polyporus oregonensis</i>	-1.5	-3.6			
<i>Fomes annosus</i>	11.0	5.1	<i>Fomes lobatus</i>	2.1	-3.8			
<i>Polyporus par-</i> <i>gamenus</i>	-4.1	4.9	<i>Polyporus versicolor</i>	13.8	-4.2			
<i>Polyporus pubescens</i>	9.2	1.1						
<i>Fomes officinalis</i>	0.0	-1.0						
<i>Poria ferruginea-fusca</i>	0.8	-3.3						
Mean	8.06	7.03		6.75	3.88		1.4	-0.3

General Discussion

The determination of lignin in the calcium lignosulphonate media is more accurate than in media prepared from waste sulphite liquor. In the latter the lignin values as measured by the β -naphthylamine precipitation method gave erratic results in the controls, there being a tendency for the lignin content to undergo an apparent increase over a period of 30 days. This drift was independent of pH changes. However, the use of media prepared with isolated calcium lignosulphonate eliminated this difficulty almost entirely. The difficulty of obtaining consistent results in lignin determinations carried out at different intervals of time still remained, however, and care had to be exercised on this point.

The survey that has included species of soil fungi such as *Alternaria*, *Fusarium*, *Phoma*, and a few species from other genera and 30 different wood destroying fungi has shown that although most of the organisms tested are capable of decomposing calcium lignosulphonate, there is a wide variation in this respect even between closely related species. In general it appears that certain of the soil fungi are more promising than the wood destroyers.

The wood destroying fungi present more cultural difficulties, are much slower in their development, and after a long period of growth many of them

tend to give an apparent increase in lignin content as measured by the β -naphthylamine precipitation method. The *Alternaria* cultures gave the greatest lignin breakdown and in those species that were retested gave very consistent results. They grow rapidly and present few difficulties in culturing on synthetic media. Certain species of *Fusarium* were capable of giving good lignin decomposition in individual tests but gave erratic results when duplicate experiments were attempted at a later date. Of the other genera tested, *Trichoderma* and *Phoma* merit further study, and it must be recognized that tests on a number of species of *Mucor*, *Aspergillus*, *Penicillium*, *Stemphylium*, and *Verticillium* might disclose the same sort of variability found with cultures of *Alternaria* and *Fusarium*. It should be borne in mind in assessing the value of any particular fungus that all cultures were grown on a standard synthetic medium in which the various nutrients were by no means present in optimum concentrations for all these organisms. The fact that in each different class of organism studied the greatest lignin decomposition was in the range of 12 to 18% indicates that factors other than the organism itself are involved. These may include nutritional or environmental factors that are not optimum, or the production of certain toxic compounds during growth which cause staling. It has been suggested by other workers (4, 8) that the cessation of lignin decomposition results from the formation of ligno-protein complexes that are inhibitory or very resistant to further microbial action. It is possible that this mechanism may account for the cessation of decomposition of calcium lignosulphonate. The fact that the precipitate obtained with β -naphthylamine in old cultures is considerably greater than in corresponding uninoculated controls suggests that the calcium lignosulphonate has combined with some metabolic products, possibly protein. On the other hand, the fact that only 12 to 18% of the calcium lignosulphonate is available to the fungus may indicate that part of the molecule only is being split off, and when this is attained further breakdown does not occur. The actual fate of lignin in nature mitigates against this explanation, but Schwalbe and Ekenstam (6) have shown that the methoxyl groups are split off by microbial action. In order to throw light on these questions further work is being carried on using a spectrographic method of analysis. A detailed investigation of the nutritional factors concerned is also being studied, using one organism grown on sodium lignosulphonate medium.

Acknowledgments

Appreciation by the authors is due to Miss M. T. Clement and Mr. A. C. Castagne for valuable assistance in the technical aspects of this work; to Dr. J. W. Hopkins for statistical treatment of the data; to the Division of Botany, Central Experimental Farm, Ottawa, and the Forest Products Laboratories, Washington, D.C., for fungous cultures.

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STUDIES ON THE RELATION OF GROWTH RATE TO WOOD QUALITY IN *POPULUS* HYBRIDS¹

By L. P. V. JOHNSON²

Abstract

Experiments were conducted on the relation of growth rate to wood quality in a series of 43 hybrid and parental trees, which involved *Populus alba*, *P. grandidentata*, and *P. tremuloides*.

Fibres in fast growth annual rings were longer on the average than those in slow growth rings from the same tree. In single annual rings, fibres of early wood were shorter and thicker than those of late wood.

Average fibre diameter of individual trees was significantly correlated in a positive manner with growth rate, but the correlation between fibre length and growth rate was well below the level of significance.

Short, thick habit of growth was significantly correlated with high density of wood, but correlations between growth rate (in terms of annual increment in volume) and wood density were insignificant.

Experimental pulp and paper tests did not reveal any very striking differences in quality between fast growing hybrid and slow growing parental trees, although there remains some doubt as to the suitability of abnormally fast growth hybrid wood for some of the higher grades of soda pulp paper.

The general, and tentative, conclusion is that the investigation revealed nothing to indicate that rapid growth is seriously detrimental to wood quality.

Introduction

It is a demonstrated fact that the growth rate of a tree affects the structure of the wood produced; for example, the more open structure of spring wood as compared to summer wood leads to the demarcation of annual rings. Therefore, since hybridity commonly introduces increased vigour of growth, the possibility of adverse wood characteristics arising from increased growth rate must be taken into account in the growing of hybrid trees.

The investigation herein reported, which is a part of a general program on forest tree breeding (2), represents an attempt to ascertain the degree to which the abnormally rapid growth (hybrid vigour) of *Populus* hybrids affects the quality of wood for pulping and other purposes. Fibre dimensions and specific gravity of the wood were used in general as the criteria of wood quality, but complete pulp and paper tests were also made on selected hybrid and parental trees.

Materials and Methods

The materials studied are as follows:

<i>P. alba</i> (A)*	2 trees
<i>P. grandidentata</i> (G)	6 trees
<i>P. tremuloides</i> (T)	3 trees
<i>P. alba</i> × <i>P. grandidentata</i> F ₁ (A × G)	24 trees
<i>P. alba</i> × <i>P. tremuloides</i> F ₁ (A × T)	8 trees
Total	43 trees

¹ Manuscript received in original form August 7, 1941, and as revised, October 2, 1941.

Contribution from the Division of Biology and Agriculture, National Research Laboratories, Ottawa. Part of a co-operative project of the Subcommittee on Forest Tree Breeding, Associate Committee on Forestry. N.R.C. No. 1025.

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* Letters in parentheses are the symbols used to designate these materials in the tabulated part of this paper.

Data on ages and dimensions of these trees are given in Table I.

Of the younger trees, ages six to seven years, those of *P. alba* grew in gravelly soil near Hull, Que., and those of all other species on a gravelly site near Masson, Que. Of the older trees, ages nine years and older, *P. grandidentata*, Nos. 1 and 2, grew near the edge of a bush adjacent to the Masson site, and all others near the Aylmer Road, Hull, Que., in low gravelly soil. Apart from *P. grandidentata*, Nos. 1 and 2, all trees were sufficiently in the open to escape suppression by other trees.

The following measurements were made on each tree: diameter, determined at stump and breast heights; height, determined by a measuring stick for trees under 35 ft. and by an hypsometer for higher trees; age, determined by the number of annual rings in the core (extracted by a standard increment borer); specific gravity, determined in a 1½-in. core removed from the butt by a specially designed borer, at a height of 18 in. from the ground. All holes were plugged with creosoted cedar dowels in order to preserve the trees for future studies.

Wood samples from spring, middle, and summer regions of average growth annual rings (and, in certain trees, fast growth and slow growth annual rings) were macerated and fibre dimensions were determined microscopically. In selecting an average growth ring, primary consideration was given to the width of the ring rather than to the year in which the ring was produced. There may be some objection to this procedure since length of fibre may vary with age of ring. However, it was considered more important, in a study dealing with growth rate, to select rings that represent average growth rate rather than rings of standard age. As it happened, average growth rings tended to occur midway between centre and bark; in six-year-old trees, for example, the average growth ring was that representing the third or, more commonly, the fourth year's growth.

Wood was macerated by immersing for 48 hr. in a freshly combined mixture of 10% nitric acid and 10% chromic acid.

All samples were permanently mounted for microscopic observations. For the most part a modification of Zirkle's (4) mounting medium was used. The procedure is as follows:

- (1) Prepare a mounting solution by mixing 4 parts glacial acetic acid, 1 part "Certo", 1 part corn syrup.
- (2) Wash macerated material.
- (3) Tease material into separate fibres in a drop of mounting solution on a slide.
- (4) Apply cover glass and seal with asphalt varnish. (A series of trial slides that were left unsealed have remained in excellent condition for several months.)

Another method of permanent mounting which, though longer than the above, proved entirely satisfactory, is as follows:

- (1) Wash macerated material.
- (2) Pass through 70%, 95%, and two changes of absolute alcohol, allowing 10 min. in each.
- (3) Tease fibres apart on slide.
- (4) Mount in Canada balsam.

Microscopic observations were made with a 16 mm. objective and a 5X ocular (50X magnification). Fibre dimensions were determined with a 5.0 mm. ocular micrometer (with the optical system used, 5.0 mm. in the ocular corresponds very closely with 1.0 mm. on the field).

In all observations on fibre length, three counts of 10 fibres each were made. As may be seen in Table IV there was very little variation between counts, and it has not been considered necessary to subject this variation to statistical analysis. The fibre length data as given in Tables II and III represent the average of three 10-fibre counts for each section of the annual ring (e.g., early wood).

In all observations on fibre diameter one count of 10 fibres was made.

Tree volumes (under the bark) were calculated from logarithmic volume tables for poplar issued by the Dominion Forest Service.

Specific gravity determinations were made by: determining the volume of trimmed, soaked (3 hr.) cores ($\frac{1}{2}$ in. in diameter, $1\frac{1}{2}$ in. long) by the water displacement method using a small specific gravity balance and weighing to the nearest 0.01 gm.; determining dry weight of the cores after drying overnight in an oven at 110° C.; and calculating specific gravity by dividing the volume in millilitres into dry weight in grams.

Experimental Results

1. Vigour Index

The product of the average annual increments in height and in diameter, herein called the vigour index, is used to express the relative vigour of growth of the "uneven-aged"* trees of the present study (Table I). Had the trees been "even-aged"**, the average annual increment in volume $\left(\frac{\text{volume}}{\text{age}}\right)$ would have been a more accurate measure of vigour since volume tables take into account the taper of the trunk (under the bark). When dealing with a series of uneven-aged trees, however, the average annual increment in volume cannot be used since it is partly dependent upon the area of cambium which, as a function of the circumference, would be disproportionately greater in large trees. In the present study the use of average annual increment in volume as an index of vigour has been restricted to the 6-yr. age class (Table V).

The vigour indices are given in Table I and are repeated as one of the variables arranged for correlative studies in Table V. Correlation coefficients between the vigour indices and other variables are given in Table V and are discussed in Subsection 6 below.

* Group comprising trees of different ages.

** Group comprising trees of the same age.

TABLE I
GENERAL DATA ON AGE, SIZE, AND VIGOUR OF TREES, AND SPECIFIC GRAVITY

Material	Age, yr.	Height, ft.	Diameter over bark, in.		Volume, cu. ft.	Average annual increment		Vigour index AB	Specific gravity
			Stump height (18 in.)	Breast height		Height, ft. (A)	Diameter, in. (B)		
A-1*	6	20	3.8	3.3	0.75	3.3	0.63**	2.10	0.459
A-2	7	22	4.3	3.7	1.0	3.1	0.61	1.92	0.502
G-1	17	34	3.2	2.8	0.84	2.0	0.19	0.38	0.369
G-2	19	40	3.9	3.4	1.38	2.1	0.21	0.44	0.361
G-3	16	46	5.4	4.6	2.76	2.9	0.34	0.98	0.430
G-4	13	60	7.7	6.5	6.70	4.6	0.59	2.73	—
G-5	19	76	13.6	11.9	22.33	4.0	0.76	3.04	0.413
G-6	15	75	10.9	9.5	16.85	5.0	0.73	3.65	—
T-1	7	25	3.6	3.1	0.79	3.6	0.51	1.82	0.437
T-2	6	29	3.9	3.4	1.05	4.8	0.65	3.14	0.424
T-3	6	29	3.8	3.3	0.99	4.8	0.63	3.04	0.363
A × G-2	6	25	5.0	4.3	1.47	4.2	0.83	3.46	0.383
-5	6	31	4.4	3.8	1.37	5.2	0.73	3.77	0.378
-13	7	20	3.8	3.3	0.75	2.9	0.54	1.54	0.365
-14	6	26	3.7	3.2	0.86	4.3	0.62	2.68	0.393
-21	6	19	3.5	3.0	0.60	3.2	0.58	1.84	—
-23	6	24	4.6	3.9	1.17	4.0	0.77	3.08	0.384
-24	6	26	3.7	3.2	0.86	4.3	0.62	2.68	0.397
-33	14	62	13.7	12.0	21.20	4.4	0.98	4.34	0.462
-35	18	75	15.5	13.5	28.95	4.2	0.86	3.59	0.400
-37	13	61	9.8	8.5	11.40	4.7	0.75	3.52	0.442
-38	15	62	11.6	10.2	15.95	4.1	0.77	3.18	0.400
-67	6	29	3.4	2.9	0.78	4.8	0.57	2.75	0.407
-72	6	27	2.9	2.5	0.52	4.5	0.48	2.16	0.344
-73	7	29	4.6	3.9	1.38	4.1	0.66	2.73	0.371
-74	6	29	4.3	3.7	1.22	4.8	0.72	3.48	0.383
-75	6	33	4.7	4.0	1.59	5.5	0.78	4.29	0.356
-76	6	34	4.8	4.1	1.71	5.7	0.80	4.54	0.338
-77	6	28	4.3	3.7	1.20	4.7	0.72	3.36	0.339
-91	7	23	3.5	3.0	0.69	3.3	0.50	1.65	0.469
-92	6	29	4.5	3.9	1.36	4.8	0.75	3.62	0.407
-93	6	27	5.0	4.3	1.56	4.5	0.83	3.74	0.366
-94	6	23	3.5	3.0	0.69	3.8	0.58	2.22	0.405
-96	6	24	4.2	3.6	1.10	4.0	0.70	2.80	0.434
-106	6	34	5.2	4.4	1.96	5.7	0.87	4.93	0.365
A × T-1	6	24	4.5	3.9	1.18	4.0	0.75	3.00	0.424
-2	6	22	2.9	2.5	0.47	3.7	0.48	1.76	—
-4	6	17	2.1	1.9	0.28	2.8	0.35	0.99	—
-5	6	21	3.9	3.4	0.82	3.5	0.65	2.28	0.440
-6	7	20	2.6	2.3	0.38	2.9	0.37	1.06	—
-12	9	55	7.1	6.1	5.52	6.1	0.79	4.83	0.355
-19	7	24	3.3	2.9	0.67	3.4	0.47	1.61	0.364
-20	7	27	3.8	3.3	0.95	3.9	0.54	2.08	0.347

* See section on Materials and Methods for explanation of symbols.

** Based on diameter at stump height.

2. Fibre Dimension in Fast Growth and Slow Growth Annual Rings of the Same Tree

Observations were made on the fibre dimensions of individual fast growth and slow growth annual rings obtained in a single core. This procedure permits a study of fibre development as related to variations in growth rate

TABLE II
COMPARISON OF FIBRE DIMENSIONS IN FAST GROWTH AND SLOW GROWTH ANNUAL RINGS OF THE SAME TREE

Material	Fast growth annual ring						Slow growth annual ring										
	Width of ann. rings, mm.	Fibre length, mm.				Fibre diameter, mm.		Width of ann. rings, mm.	Fibre length, mm.				Fibre diameter, mm.				
		Early season wood	Mid-season wood	Late wood	Av.	Early wood	Mid-season wood		Late wood	Av.	Early season wood	Mid-season wood	Late wood	Av.			
G-4	11	0.96*	1.01	1.03	1.00	0.026**	0.029	0.024	0.026	3	0.83	0.96	1.02	0.94	0.027	0.024	0.024
G-5	11	0.97	0.99	0.99	0.98	0.027	0.027	0.025	0.026	5	0.95	0.96	0.96	0.96	0.028	0.027	0.027
A × G-2	13	0.78	0.95	0.98	0.90	0.027	0.026	0.026	0.026	3	0.52	0.53	0.63	0.56	0.025	0.025	0.025
A × G-33	15	0.90	1.00	1.03	0.98	0.024	0.025	0.024	0.024	6	0.94	0.97	1.04	0.98	0.022	0.023	0.022
A × G-35	11	0.92	1.01	1.00	0.98	0.030	0.029	0.028	0.029	3	0.72	0.76	0.84	0.77	0.026	0.026	0.026
A × G-38	9	1.02	1.06	1.09	1.06	0.027	0.026	0.024	0.026	3	1.01	1.02	1.07	1.03	0.026	0.023	0.024
A × G-93	12	0.77	0.98	0.97	0.91	0.032	0.030	0.030	0.031	4	0.76	0.79	0.79	0.78	0.034	0.034	0.034
A × G-106	23	0.96	0.94	0.99	0.96	0.027	0.025	0.024	0.025	10	0.79	0.98	1.01	0.93	0.028	0.026	0.026
A × T-12	14	0.70	0.99	1.05	0.91	0.026	0.022	0.021	0.023	4	0.88	0.89	0.91	0.89	0.024	0.023	0.023

* Each figure on fibre length is based on 30 fibres, thus the average of the three samples from each ring is based on 90 fibres.

** Each figure on fibre diameter is based on 10 fibres, thus the average of the three samples from each ring is based on 30 fibres.

caused solely by environmental factors—hereditary factors being constant in the somatic tissues of a single individual.

The data presented in Table II indicate that fibre length tends to be greater in fast growth annual rings. Of the nine trees examined, the fast growth annual rings of eight had longer fibres than had corresponding slow growth annual rings. In the remaining tree ($A \times G-33$) the fibre length was the same in both fast growth and slow growth rings. The difference in fibre length could be called striking only in three trees ($A \times G-2$, $A \times G-35$, and $A \times G-93$). The analysis of variance method applied to fibre length data in fast and slow growth annual rings gave an F value well above the 5% level of significance.

As regards fibre diameter, the data do not indicate any consistent relation between this character and rate of growth in individual annual rings. The F value obtained was not significant.

3. *Fibre Dimensions of Early, Midseason, and Late Wood of the Same Annual Ring*

It is a well known fact that in trees the xylem elements produced in the spring differ in diameter (as shown by cross sections) from those produced later in the growing season. It is also generally thought that the rate of growth is relatively more rapid in the spring and early summer than in the later part of the growing season. This suggests a relation between cell size and growth rate and has led to observations on dimensions of fibre in wood produced in the early, middle, and late periods of the growing season, respectively. The results are given in Tables II and III.

The results may be stated briefly as follows: fibres produced in the spring have a strong tendency to be relatively thicker and shorter than those produced late in the growing season. Data in Tables II and III show that in the 27

TABLE III

COMPARISON OF FIBRE DIMENSIONS OF EARLY, MIDSEASON, AND LATE WOOD OF AN AVERAGE GROWTH ANNUAL RING

Material	Fibre length, mm.				Fibre diameter, mm.			
	Early wood	Mid-season wood	Late wood	Av.	Early wood	Mid-season wood	Late wood	Av.
$A-1$	0.84	0.97	0.87	0.89	0.026	0.025	0.026	0.026
$G-2$	0.85	0.98	1.08	0.97	0.023	0.023	0.022	0.023
$G-5$	0.93	0.98	0.97	0.96	0.029	0.029	0.026	0.028
$T-3$	0.61	0.68	0.69	0.66	0.030	0.026	0.026	0.027
$A \times G-35$	0.96	0.96	1.04	0.99	0.036	0.032	0.031	0.033
-72	0.79	0.91	0.99	0.90	0.025	0.026	0.025	0.025
-93	0.85	0.92	0.88	0.88	0.025	0.026	0.026	0.026
-94	0.63	0.72	0.90	0.75	0.025	0.027	0.025	0.026
-106	0.67	0.76	0.78	0.74	0.025	0.026	0.024	0.025

NOTE: The footnote to Table II applies also to Table III.

annual rings (nine each of fast, slow, and average growth rings) examined, fibres of early wood were shorter than those of late wood, those of midseason wood being, in general, of intermediate length. The *F* value (analysis of variance) obtained for fibre length in early, midseason, and late wood attained the 1% level of significance for data in each table. In average diameter, fibres of early wood were greater than those of late wood in 20 out of 27 rings, with diameters equal in six rings—there being only one ring in which late wood fibres were of greater diameter. Here again fibres of midseason wood tended to be intermediate. The *F* value obtained for fibre diameter in early, midseason, and late wood attained the 1% level of significance for combined data of fast and slow growth annual rings (Table II), but was not significant for average growth rings (Table III).

4. *Fibre Dimensions of Hybrids and Parents of Different Growth Rates.*

From observations on fibre dimensions in early, midseason, and late wood (Tables II and III) it was found that the fibres in midseason wood were, in general, intermediate in dimensions and corresponded fairly closely to the average for the annual ring concerned. Therefore, in making observations on the relatively large number of remaining trees it was considered sufficient to use only the midseason wood.

In Table IV, data are given on the average length and average diameter of fibres, taken from the midseason part of average growth annual rings, of a series of 43 hybrid and parental trees which differ markedly in vigour of growth. The variability between counts on fibre length in a given annual ring is very slight, the greatest difference being in G-3 where counts ranged from 0.88 to 0.97 mm. The standard error is 0.0103. Variability in vigour indices is much greater, however, being from 0.38 to 3.65 among the parents and from 0.99 to 4.93 among the hybrids.

These data are included in the correlative studies described in Subsection 6.

5. *Specific Gravity Studies*

Specific gravity determinations were made on cores of wood ($\frac{1}{2}$ in. in diameter) from the trunks (18 in. above ground) of 37 hybrid and parental trees. Results are given in Table I.

Since the removal of large cores might prove injurious, an attempt was made to establish a relation between the specific gravity of core wood and of wood from typical lower branches of the same tree. The coefficient of correlation (*r*) calculated for the data obtained fell well below the 5% level of significance (Table V). It was concluded therefore that the specific gravity of branch wood and that of trunk wood of the same tree are not sufficiently related to permit the substitution of the former for the latter in the present study.

Specific gravity determinations were also made on samples (disks) taken at 4-ft. intervals along the trunks of a number of trees that were cut down. The following data, which represent the specific gravity of successive disks taken at 4-ft. intervals commencing at the base, are typical of the results

TABLE IV

FIBRE DIMENSIONS OF HYBRIDS AND PARENTS OF DIFFERENT GROWTH RATES

Material	Vigour index	Midseason wood of average growth ring	
		Average* fibre length, mm.	Average** fibre diameter, mm.
A-1	2.10	0.97 \pm 0.01	0.025
A-2	1.92	0.77	0.027
G-1	0.38	0.95	0.024
G-2	0.44	0.98	0.023
G-3	0.98	0.93	0.026
G-4	2.73	1.02	0.028
G-5	3.04	0.98	0.029
G-6	3.65	0.98	0.029
T-1	1.82	0.74	0.026
T-2	3.14	0.68	0.024
T-3	3.04	0.68	0.026
A \times G-2	3.46	0.88	0.026
-5	3.77	0.78	0.038
-13	1.54	0.83	0.026
-14	2.68	0.97	0.026
-21	1.84	0.85	0.023
-23	3.08	0.93	0.027
-24	2.68	0.95	0.031
-33	4.34	0.94	0.024
-35	3.59	0.96	0.032
-37	3.52	0.97	0.028
-38	3.18	1.11	0.033
-67	2.75	0.90	0.026
-72	2.16	0.91	0.026
-73	2.73	0.93	0.028
-74	3.48	0.82	0.031
-75	4.29	0.92	0.030
-76	4.54	0.94	0.028
-77	3.36	0.82	0.037
-91	1.65	0.81	0.023
-92	3.62	0.93	0.034
-93	3.74	0.92	0.026
-94	2.22	0.72	0.027
-96	2.80	0.86	0.026
-106	4.93	0.76	0.026
A \times T-1	3.00	0.93	0.026
-2	1.76	0.93	0.029
-4	0.99	0.75	0.022
-5	2.28	0.94	0.028
-6	1.06	0.90	0.031
-12	4.83	0.95	0.030
-19	1.61	0.85	0.030
-20	2.08	0.80	0.027

* Average based on three counts of 10 fibres each.

** Average based on one count of 10 fibres.

obtained; G-2: 0.361, 0.355, 0.344, 0.326, 0.338, 0.352, 0.342, 0.360, 0.373, 0.380; A \times G-106: 0.365, 0.306, 0.293, 0.293, 0.275, 0.280, 0.286, 0.327.

These results show that specific gravity is relatively high at the base, gradually becoming lower until a height of 15 to 20 ft. is reached, and then gradually increasing with each succeeding interval to the top of the tree.

TABLE V

CORRELATION COEFFICIENTS (r) CALCULATED FOR DIFFERENT COMBINATIONS OF VARIABLES

Combination of variables	Age group	Number of trees	r
Average annual increment in height with:			
average annual increment in diameter	All-age	43	.75**
vigour index	All-age	43	.92**
specific gravity	All-age	37	-.28
average length of fibre	All-age	43	-.00
average diameter of fibre	All-age	43	.43**
Average annual increment in diameter with:			
vigour index	All-age	43	.92**
specific gravity	All-age	37	.06
average length of fibre	All-age	43	.12
average diameter of fibre	All-age	43	.34*
Vigour index with:			
height/diameter	All-age	43	-.43**
volume/age	6-yr.	24	.59**
specific gravity	All-age	37	-.16
average length of fibre	All-age	43	.09
average diameter of fibre	All-age	43	.40**
Height/diameter with:			
specific gravity	All-age	37	-.41**
average length of fibre	All-age	43	-.04
average diameter of fibre	All-age	43	-.14
Volume/age with:			
specific gravity	6-yr.	21	-.22
average length of fibre	6-yr.	24	.01
average diameter of fibre	6-yr.	24	.27
Specific gravity with:			
average length of fibre	All-age	37	-.02
average diameter of fibre	All-age	37	-.25
Average length of fibre with:			
average diameter of fibre	All-age	43	.17
Core branch (specific gravity)	All-age	37	.26

* Denotes attainment of 5% level of significance.

** Denotes attainment of 1% level of significance.

6. Correlation Between the Different Variables under Study

The variables used for correlative studies include primary observational factors, such as tree dimensions, fibre dimensions, and specific gravity, and also secondary factors, such as vigour index, height-diameter ratio, and volume-age ratio.

Table V gives the values of the correlation coefficient (r) calculated for 24 pairs of variables, together with data on the numbers and age grouping of trees involved.

There is a very high correlation between average annual increments in height and a diameter, which was to be expected. The correlation coefficients between these factors and the vigour index, which is a function of them, are, of course, also high.

Fibre diameter is positively correlated with average annual increments in height (1% level of significance) and in diameter (5% level), and with vigour index (1% level).

It will be noted that the values of r for fibre length in corresponding combinations are very low. This means that as growth rate is increased the diameter, but not the length, of fibres is significantly increased.

It is important to note how this increase in fibre diameter affects values of r where specific gravity is concerned. It is found that r has a negative value in all combinations, except that with average annual increment in diameter of trunk, but only in the combination with the height-diameter ratio does it reach significance (1% level). This means that as trees tend to become tall and slender (as opposed to short and thick) the specific gravity of the wood tends to decrease.

The remaining significant correlation involves the two general measures of growth rate, vigour index, and volume-age ratio. The value of r is well above the 1% level of significance. As indicated in Subsection 1 above, the volume-age ratio is a more desirable measure of growth vigour than the so-called vigour index. However, since it was necessary to use the vigour index it is reassuring to know that it is highly correlated with the volume-age ratio.

7. *Experimental Pulp and Paper Tests of a Slow Growing Parent and a Fast Growing Hybrid*

It was considered important to investigate the differences in pulp (soda) and paper quality that might be found in wood samples derived from a relatively slow growing parent and from an abnormally rapid growing hybrid. Accordingly, entire trunks of two such trees were sent to the Pulp and Paper Division, Forest Products Laboratory, Montreal, for testing. The report of the Laboratory, which includes comparisons with commercial poplar pulp, is summarized and discussed in the following paragraphs.

(a) *Description of Materials*

The trees used in the pulp and paper tests were also used in the general studies, the *P. grandidentata* parent being *G-2* and the *P. alba* \times *P. grandidentata* hybrid being *A* \times *G-106*. The growth of *G-2* had been considerably suppressed by surrounding bush and at the time of cutting, when it was 19 years old, it was 40 ft. in height and 3.9 in. in diameter. The tree, *A* \times *G-106*, grew near *G-2* but under less competitive conditions; it grew very rapidly being 34 ft. in height and 5.0 in. in diameter at six years of age. For further data on *G-2* and *A* \times *G-106* see Tables I to IV inclusive.

It was considered that, since these trees differed so greatly in growth rate, the tests should indicate whether any marked peculiarities in pulp or paper quality might exist in the wood of abnormally rapid growing poplar hybrids.

(b) *Chemical Analysis of the Wood*

No appreciable difference in chemical composition was indicated by wood analysis. *A* \times *G-106* was slightly higher than *G-2* in lignin, but was, consequently, somewhat lower in cellulose.

(c) *Screen Classification of Bleached Soda Pulp*

The screen classification showed that G-2 compared very closely with commercial poplar pulp in fibre length in both beaten and unbeaten conditions. The fibres of $A \times G-106$, however, were shown to be appreciably longer than those of either G-2 or average commercial pulp; but not longer, however, than some of the longer-fibred commercial samples of poplar pulp.

(d) *Strength of Unbeaten and Unbleached Soda Pulp*

$A \times G-106$ proved to be appreciably the stronger, both in slush and air-dry pulp, for burst factor and breaking length (length at which strip breaks of its own weight); it also gave a much higher percentage of stretch. In G-2 the bulk in ml. per gm. was considerably greater. In tear ratio the two samples were about equal.

(e) *Strength of Beaten and Bleached Soda Pulp*

Tappi beater tests (various periods of beating from 5 to 60 min.) of bleached soda pulp showed that $A \times G-106$ was consistently stronger than G-2 or commercial pulp for burst factor and breaking length, and gave consistently higher percentages of stretch. At the start of the beating, $A \times G-106$ gave a rather high tear ratio but after a few minutes of beating it registered a somewhat lower value than the other two samples. In bulkiness, $A \times G-106$ was considerably below the other two samples for all beating periods.

(f) *Optical Properties*

Commercial poplar pulp was consistently better than that of G-2 or $A \times G-106$ in brightness, reflectance, and printing opacity of the paper. This superiority was slight at the beginning of the beating period but became considerable after 60 min. The paper from G-2 was slightly better than that from $A \times G-106$ in these optical qualities.

(g) *Discussion Regarding the Suitability of $A \times G-106$ for Pulp Manufacture*

Before discussing the results it must be recognized that the present pulping tests do not provide an adequate basis for positive conclusions. Obviously, portions of several trees rather than a single trunk would be necessary to provide a representative sample, either of the hybrid or the parent. Further, in the present test the age of the hybrid tree was less than one-third that of the parent and probably less than one-fifth that of trees used in commercial pulping.

However, until better data are available, there are a few things that may be said with some safety about the present results.

For soda pulp paper, the pulp of $A \times G-106$ appears to have one main peculiarity that might cause discrimination against it—that is, the condition of being stronger and less bulky than the pulp of ordinary mill-run poplars. Shorter, weaker fibres give the pulp a greater bulkiness, which in turn gives the desired formation and printing quality to the higher-grade soda pulp papers. It is possible that the pulp from trees such as $A \times G-106$ might not meet the standards demanded for these papers.

It must be remembered, however, that a large part of the poplar wood pulped in Canada is utilized, in the sulphite process, as a mixture with spruce and balsam fir. In sulphite pulp, unlike soda pulp, a long, strong fibre is desired, and the proportion of poplar wood that may be mixed with the other pulpwoods is determined by the degree of weakening occasioned by the shorter poplar fibres. It follows, therefore, that if a rapid growing hybrid poplar had longer fibres than ordinary poplars, a correspondingly greater proportion of its wood could be mixed with other pulpwoods in the manufacture of sulphite pulp. This would lead to a greater demand for poplar pulpwood.

It may be, therefore, that there is a place in the pulp industry for both short- and long-fibre types of poplar.

It should be noted that results of the present studies (Subsection 6) do not establish any marked relation between growth rate and fibre length in a series of 43 trees.

General Discussion and Conclusions

In the hybrid poplars under study, rapid growth is highly desirable—provided wood quality is not adversely affected. Therefore, to know the true commercial value of rapid growth (hybrid vigour) the relation between growth rate and wood quality must be known.

The data on fibre length in individual fast and slow growth annual rings from the same tree show that there is a marked tendency for fast growth rings to have the longer fibres. On the other hand, data on seasonal wood of single annual rings demonstrate unmistakably that early season wood has shorter fibres than late season wood. If, as seems to be the accepted view, wood is laid down faster in the early part of the growing season, these two types of data are in opposition with respect to the implied relation between growth rate and length of fibre produced. In the data from individual trees, statistically significant relation between fibre length and growth rate could not be demonstrated.

From the practical point of view, the conclusion from studies on fibre length must be: that it has not been possible to prove the existence of any consistent relation between fibre length and growth rate that might be reflected in the wood quality (as affected by fibre length) of rapid growing trees. This is in agreement with the results of a study on white spruce reported by Lee (3) who concluded that rate of growth had little effect on fibre length.

Data on fibre diameter from individual trees show a highly significant positive correlation with growth rate. Data from seasonal wood of single annual rings demonstrate a marked tendency for fibres of early season wood to be of larger diameter than those of late season wood. If it is assumed that growth is fastest in the spring, this result is in agreement with results from individual trees.

Since there would be less cell wall material per unit volume in tissue in which the fibres are relatively large in diameter, there is reason to expect that large diameter fibres would lead to a reduction in specific gravity of the

wood. In the present study, however, this relation was not strong enough to produce significant correlation coefficients when specific gravity was paired with either fibre diameter or growth rate. In so far as the present study is concerned, therefore, it can only be said that a relation between fibre diameter and growth rate was established, but that no definite information was obtained on how this relation might be reflected in the wood density of rapid growing hybrids.

In this connection, it is useful to cite the recent work of Hale and Prince (1) in which it was found that rapidly grown wood of spruce and balsam fir was of lower density than slowly grown wood. In their summary these authors make a statement that has a direct bearing on the practical aspects of the present discussion: "... although rapidly grown wood . . . is light in weight and the slowly grown wood relatively heavy, the rate of wood production of rapidly grown trees on a basis of total weight far exceeds the amount produced by slow growing trees. Therefore, from the point of view of the paper manufacturer, who requires large quantities (by weight) of wood produced in the shortest practicable time, fast growing trees will be essential, and extremely slow growing stands, however desirable the quality of their wood, will tend more and more to be uneconomical for pulpwood production."

The results of the pulp and paper tests have already been discussed at considerable length, and it remains merely to state that the tentative conclusion reached was that there appeared to be good reason to believe that rapid growing hybrid poplars could be utilized to good advantage either as soda or sulphite pulp or both. As a matter of fact, it may be possible for the breeder to produce long- and short-fibred trees for utilization as sulphite and soda pulp, respectively.

The general conclusion, which must be a tentative one, to be drawn from these studies is that nothing has been found that would support a contention that abnormally rapid growth is seriously detrimental to wood quality. For the time being, therefore, breeding work on the production of rapid growing forest trees will proceed with some assurance that rapid growth and good quality wood are not incompatible. It is hoped that further assurance in this connection may be forthcoming from future experiments.

Acknowledgments

The author gratefully acknowledges the assistance rendered by the Forest Products Laboratories of the Dominion Forest Service; by Dr. J. W. Hopkins, Statistician at the National Research Laboratories; and by Lieut. R. A. Packman, formerly of Ontario Agricultural College.

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THE DEVELOPMENT AND STRUCTURE OF THE CONIDIA OF *ERYSIPHE POLYGONI* DC. AND THEIR GERMINATION AT LOW HUMIDITY¹

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Abstract

The germination of the conidia of *Erysiphe Polygoni* DC. takes place through a range of relative humidity from approximately zero to 100% and, therefore, independently of the moisture content of the surrounding atmosphere. In germinating thus, they differ from the spores of some other erysiphaceous fungi and of non-erysiphaceous fungi in general.

In *E. Polygoni*, the conidium is cut off from the conidiophore by a ring of wall material which is added to inwardly until a perforate disk is formed. Later, the pore is closed and the mature conidium remains attached to its conidiophore only by a minute papilla. The conidia have never been observed to germinate *in situ*, and they are passively discharged.

The conidium wall is relatively impervious to water, stain passing into the spore only at the papillate end. Assuming the wall to be relatively impervious to gases also, an explanation is offered for the mechanism of germination of the conidia when they are dislodged from their conidiophores and allowed to fall on dry slides. The papilla provides a permeable spot in the spore wall. It is not exposed until after the spore has been detached. Upon exposure to air, the papilla allows carbon dioxide to pass out from the protoplast and oxygen to pass in, causing respiration and other germination processes to begin.

Evidence in support of this suggestion is presented. When freshly detached conidia were held in an atmosphere containing 10% carbon dioxide, germination was checked. These conidia germinated perfectly when removed from the carbon dioxide. Germination was similarly checked by holding the spores in an atmosphere of nitrogen.

No shrinkage of the conidia during germination was observed, but shrivelling and collapse take place when death is imminent.

Introduction

In 1936, Yarwood (23) stated that the conidia of *Erysiphe Polygoni* DC. and certain other powdery mildews are capable of germinating at low relative humidity, even approaching zero.

It has been recognized that spores of fungi in general germinate best under conditions of high humidity and that, in many instances, they must be in actual contact with water. Yarwood's work, therefore, presented a problem of paramount importance, the solution of which might be expected to throw light upon the epidemiology and control of the powdery mildews and upon their physiology. Yarwood did not explain why mildew conidia are able to tolerate low humidity, and no confirmation of his observations has been offered up to the present.

The senior author, in 1937, carried out preliminary experiments with conidia of *E. Polygoni* taken from cabbage leaves. This work was not published, but

¹ Manuscript received August 15, 1941.

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it showed clearly that Yarwood had not been mistaken: a high percentage of germination of conidia was obtained under conditions of extremely low humidity. Encouraged by this finding, the writers undertook a further investigation of the peculiar behaviour of the mildew conidia in an effort to corroborate Yarwood's work and to discover why mildew spores tolerate low humidity.

Historical

Prior to the publications of Yarwood (22, 23), there are few references in the literature containing data sufficiently relevant to the present problem to warrant detailed review in this paper.

Early workers concerned themselves chiefly with the taxonomy and morphology of the Erysiphaceae. Regarding the germination of conidia in the Erysiphaceae, the brothers Tulasne (20) merely mention that germinating spores were seen frequently in the material examined.

Neger (16), in 1902, studied the germination of mildew spores under different environmental conditions and showed that certain characteristics are typical of the germ tubes of each species. Each of these characteristics (length of germ tube, manner of branching, etc.) varies over a wide range and is affected by light and temperature. Similar views were expressed by Hammarlund (12) and Corner (8).

It has been observed repeatedly that the conidia of powdery mildews germinate only very scantily in water. Among those who have reported this fact in a large number of mildew species are Neger (16), Foëx (10), Sawada (18), Woodward (21), Graf-Marin (11), and Corner (8). According to Graf-Marin, Corner, and others, any germ tubes that are able to make their appearance in water may be longer than those produced in moist air. Sawada (18) reported that the conidia of some species of *Phyllactinia* and *Uncinulopsis* are killed by immersion in water.

As reported by the workers mentioned above, the optimum temperature for the germination of most powdery mildew conidia is about 25° C. *Uncinula necator* (Schw.) Burr. (*Oidium Tuckeri* Berk.) (24) and *Sphaerotheca pannosa* (Wallr.) Lev. (12) are exceptional, the optimum temperatures of germination being 25° to 28° and 30° C., respectively.

A considerable amount of investigation has been reported on the process of the development of the conidia. Brief reference to this work is made for comparison with the studies on the formation of conidia in *E. Polygoni* described in this paper.

It has been pointed out by Beeley (2), Blumer (5), and Hammarlund (12), that dry conditions favour the development of powdery mildew mycelium and encourage the production of conidia.

Massee (15) stated that the liberation of the conidia of *Sphaerotheca Humuli* (DC.) Burr. on vegetable marrow occurs chiefly at night. Hammarlund (12) reported that, in *Erysiphe communis* Auct. Amer. (*E. Polygoni*), from one to six

conidia are developed on each conidiophore each day and that the conidia are forcibly discharged.

In species that produce their conidia in chains, Foëx (9) showed that the basal cell and the one above it divide to produce the conidia (*S. Humuli*). Blumer (4) demonstrated that in *Erysiphe Cichoracearum* DC. and *Sphaerotheca* spp., the basal cell is the one from which conidia are formed.

Hammarlund (12) stated that, in *E. communis*, humidity affects the length of the chain of spores produced. In this fungus there is usually only one conidium formed at a time on each conidiophore. However, under moist conditions, chains of spores may be formed, the length of the chain increasing as the humidity increases.

In 1936, Yarwood (22) described, in *E. Polygoni* on red clover, a diurnal cycle which is manifest in the maturation of the conidia and their germinability. Each conidiophore forms one conidium per day and the spore is liberated passively about noon. Yarwood obtained the highest percentage of germination of mildew conidia when they were removed from clover from midday to four o'clock. Germinability decreased with the onset of darkness and reached a minimum in the early morning.

It was shown that light has a definite stimulatory effect upon germination: conidia collected during the high phase of the germination cycle germinated almost as well in darkness as in light, whereas germination of conidia collected in the low phase was greatly stimulated by light. In field experiments, inoculations with clover mildew spores made during the light portion of the day were more successful than inoculations made at night.

Recently, Childs (7) has demonstrated a similar cycle for a number of mildews including several that produce their spores in chains.

Working with *E. Polygoni* from various host plants, *E. Cichoracearum* from sunflower, and *S. pannosa* from rose, Yarwood (23) found that, at 22° C., germination of mildew conidia was good at relative humidities ranging from 100% to approximately zero, in many experiments the percentage germination at zero being as high as at 100%. Although as much as 65% germination was observed at zero relative humidity, the conidia were found to have shrivelled and died at the end of 30 hr., whereas at higher humidity a much smaller proportion shrivelled. In other words, the conidia tolerated low humidity to the extent of being able to germinate but ultimately more of them were injured by dry air than by moist. The tolerance of low humidity was also shown to decrease with increase of temperature.

Yarwood stated that the volume of the conidia decreases as much as 24% during germination. In contrast, conidia of several other fungi that did not germinate without being in actual contact with water increased greatly in volume during germination.

In field experiments, best infection of clover, bean, cabbage, barley, cantaloupe, *Delphinium*, and mustard was obtained when the inoculation was made at low humidity.

Yarwood's conclusion may be quoted: "Though, under certain conditions, better development of *E. Polygoni* has resulted under conditions of low rather than those of high humidity, the writer believes that high humidity is not, in itself, markedly injurious to the powdery mildews studied Rather, the writer believes that the forms studied can develop luxuriantly over a wide range of relative humidities and that they are especially well adapted, in contrast with most parasitic fungi, to very dry atmospheric conditions."

Not all species of powdery mildew tolerate low humidity. Yarwood found that *E. Polygoni* from mustard, *E. graminis* DC. from barley, *E. Cichoracearum* from sunflower, and *S. pannosa* from rose, were considerably less tolerant than other species and strains tested.

Berwith (3) studying *Podosphaera leucotricha* (E. & E.) Salm., the apple powdery mildew, claimed that high humidity is necessary for the germination of the conidia and the infection of apple seedlings, while Hashioka (13) showed that, in Formosa, the conidia of *Sphaerotheca fuliginea* (Schlecht.) Poll. on cucurbits do not germinate under conditions of low humidity. He believed, however, that the most abundant formation of conidia occurs at low humidity.

From the evidence presented above, it may be concluded that at least certain members of the Erysiphaceae are remarkable in that their conidia possess the ability to germinate under conditions of low humidity, in this respect being unlike other fungi. So far as the writers are aware, no explanation for this peculiar characteristic of the powdery mildews has been offered. The results of the investigations recorded in this paper throw some light upon the problem, although they cannot be said to present its complete solution.

Materials and Methods

The organism most extensively used was *Erysiphe Polygoni* DC., obtained from knotweed (*Polygonum aviculare* L.) on the Fort Garry campus of the University of Manitoba during the summers of 1939 and 1940. Other mildew species were also examined; they will be mentioned later.

The mildewed leaves were gathered at midday when, as shown by Yarwood (22), the spores were found to exhibit maximum germinability. Germination tests were carried out immediately after collection, the conidia being placed in diffuse light in the laboratory at a temperature of 20° to 23° C. Clean cover slips were placed at the bottom of a large crock, and mildewed leaves were shaken to allow spores to settle on the cover slips assuring a uniform distribution.

Large Petri dishes sealed with vaseline served as chambers to provide a range of relative humidity from zero to 100%. Each dish was half-filled with a saturated salt solution according to the directions given by Spencer (19, pp. 67, 68). The cover slips bearing spores were supported above the solutions on glass rings. It was thought that, even in the presence of a humidity-regulating solution, the air in a small chamber might not be uniform as to

its moisture content. Yarwood placed the Petri plates on an incline and rotated them so that the solution and the air in the chamber were agitated. Under these conditions, he obtained results entirely comparable to those obtained when plates were not rotated. In the present investigation, at the lowest humidities, a further precaution was taken to make certain that the air in which the conidia germinated was actually extremely dry. Air was bubbled through three tall wash towers containing concentrated sulphuric acid before being admitted to the germination chamber.

Each germination test was based on a count of 200 conidia. In some material, it was noticed that a very small proportion of the spores had germinated before the experiment had begun. In no instance was this proportion higher than 1% and it was finally neglected in making the count of conidia germinated at the end of the test. Some shrivelled (doubtless dead) spores were nearly always found in freshly gathered material, but never more than 3% of these in a sample. The germination percentages were based on counts of all conidia in the fields examined with the microscope.

In testing the germination of the conidia in various gases, the spores on cover slips were placed in the germination chamber into which the desired gases could be admitted. As far as possible connections in this apparatus were ground glass; pure gum rubber was used only where necessary. A description of the apparatus is given by Neufeld (17).

While studying the structure of the conidiophore of *E. Polygoni*, it was found that internal details were revealed by the use of a very simple staining method. Infected leaves were folded so that the conidiophores projected beyond the leaf. The leaves were then immersed for five minutes in a 5% solution of iodine in potassium iodide and were then examined in water on a slide. The protoplast was fixed by this treatment and stained dark brown. Also, septa were especially distinct in material so treated.

Development of the Conidiophore and Conidium of *Erysiphe Polygoni*

Because the mode of formation of the conidia has a possible bearing on the mechanism of germination, material of *E. Polygoni* on *Delphinium* was studied both when living and after being stained.

The young conidiophore is at first terete, with a blunt apex (Fig. 1a), and is aseptate. A slight swelling appears in the apical region and, within the cell wall, a ring of shiny material forms, becoming more and more clearly defined until it is almost as readily distinguished as the annular thickenings in angiosperm vessels (Fig. 1b). Considering the simple nature of the stain, it was surprising to find that excellent examples of various stages in the development of the septum were visible in every preparation.

Material is apparently added to the ring toward the inside until a disk is formed (Figs. 1c and 1e). The centre of the disk remains perforate until the conidium is mature and provides a pore which, although not discernible in

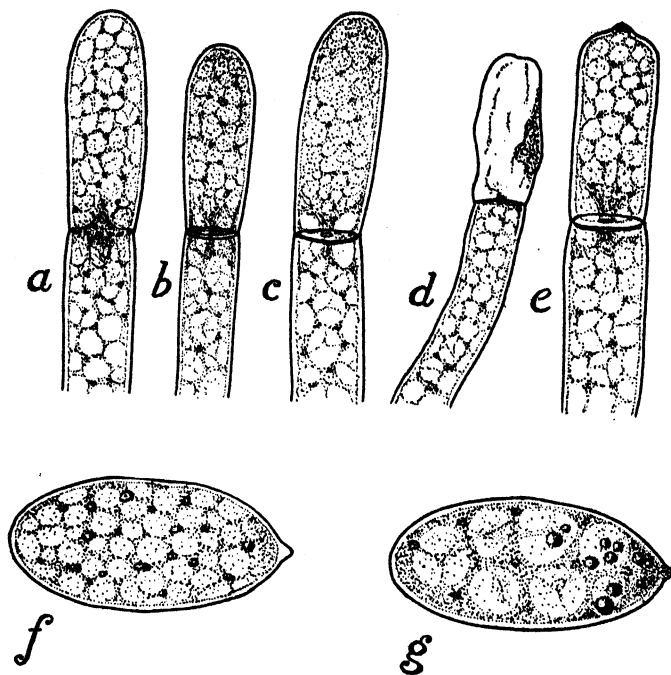


FIG. 1. *Erysiphe Polygoni*: a, b, c, e, stages in the development of the perforate septum of the conidiophore; the vacuolate appearance of the cytoplasm is probably not due to the presence of true vacuoles (see text); d, conidiophore bearing a dead conidium and showing plugging of the septal pore as a result of the death of the conidium; f, conidium as seen in water showing large globules in the protoplast and smaller refractive bodies; g, same conidium after standing two hours in neutral red stain showing enlargement of globules, and heavily stained refractive bodies near papillate end. $\times 1100$.

living material, shows up splendidly when the conidiophore is immersed in iodine. Pores were also seen in septa of mycelial cells although these were not as readily found as the pores in the conidial septa.

The nature of the septum in fungi has been fully discussed by Buller (6). The pore in the septum of the conidiophore of *E. Polygoni* seems similar to what Buller has described for other fungi. To the writers' knowledge, no demonstration of the septal pore has hitherto been given for a member of the Erysiphaceae. Allen (1) stated that in *E. Polygoni* she believed the adjoining mycelial cells to be connected by cytoplasmic strands and that cytoplasmic streaming occurs in the mycelium. In a fungus such as this, the biological importance of septal pores is very great. Food material is absorbed only by the haustoria. Septal pores make possible continuity of the cytoplasm from the haustoria to the spore-bearing hyphae. Doubtless there is a constant flow of cytoplasm from the haustoria to the conidiophores. Buller (6) has demonstrated that cytoplasmic streaming occurs in many kinds of fungi and the writers endeavoured to observe it in living mildew mycelium. They were unable to do so, but this may have been because it was necessary to remove

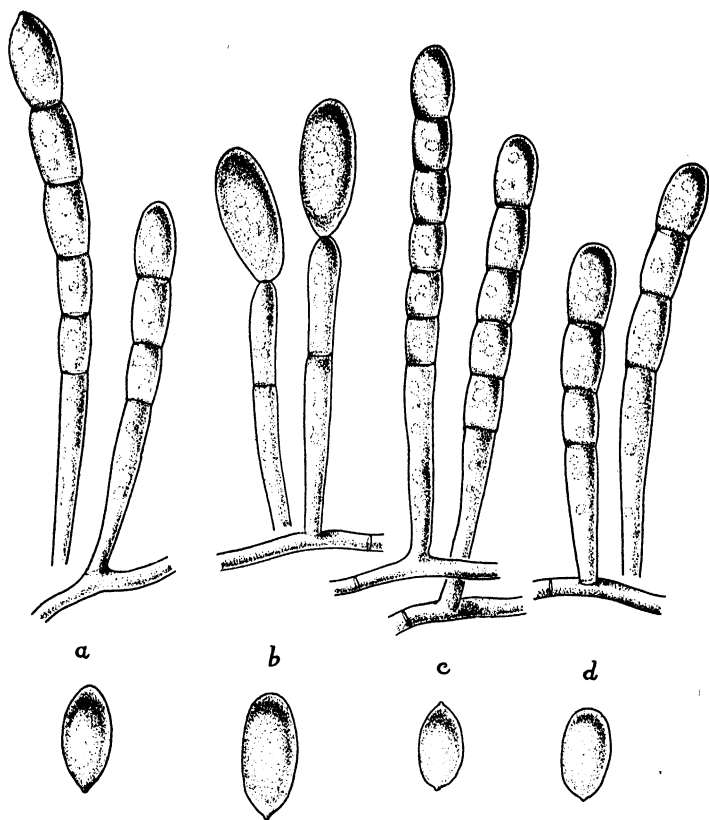


FIG. 2. Various mildew conidia showing variation in size and shape of spores: a, *Erysiphe graminis*; b, *Erysiphe Polygoni*; c, *Sphaerotheca Humuli*; d, *Sphaerotheca Humuli* var. *fuliginea*. $\times 500$.

the mycelium from the host leaf in order to study it with the high power of the microscope. In view of the prominence of septal pores in the mycelium of *E. Polygoni*, the writers do not doubt that streaming occurs.

How the perforate septum (Fig. 1c) undergoes modification as the conidium matures, the writers did not determine. The end cell of the conidiophore swells and becomes the conidium, which, when mature, remains attached to the conidiophore only by a minute point of contact (Fig. 3e).

A detached conidium of *E. Polygoni* bears a minute shiny papilla at one end (Fig. 3d) and the parent conidiophore is papillate after the conidium has been dislodged (Fig. 3g). Such careful observers as the Tulasnes (20) did not illustrate the papilla, and other workers do not appear to have realized its importance. The present writers believe this papilla to have special significance in the germination process, as will be brought out later in this paper.

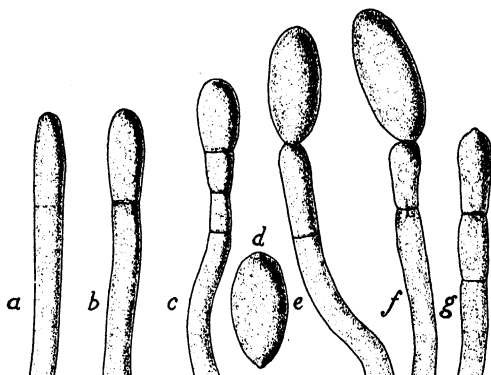


FIG. 3. *Erysiphe Polygoni*: stages in the development and abstriction of the conidia. $\times 500$.

The conidium first formed on a young conidiophore bears only one papilla which is developed at its proximal end. Since a papilla is also formed on the conidiophore at the end attached to the conidium, the second spore formed on the same conidiophore bears two papillae, one at either end. In a mass of spore material, therefore, one finds some conidia with one papilla and some with two.

The papilla appears as a highly refractive structure, the conidium wall being thickened in the papilla region. The papilla is probably not an ordinary germ pore because the germ tube always emerges slightly to one side and not through it (Fig. 4b).

As stated above, Hammarlund (12) believed that the conidia of *E. Polygoni* are forcibly discharged. Yarwood denied this (22) stating that they are passively liberated. To settle this point, the writers made careful observa-

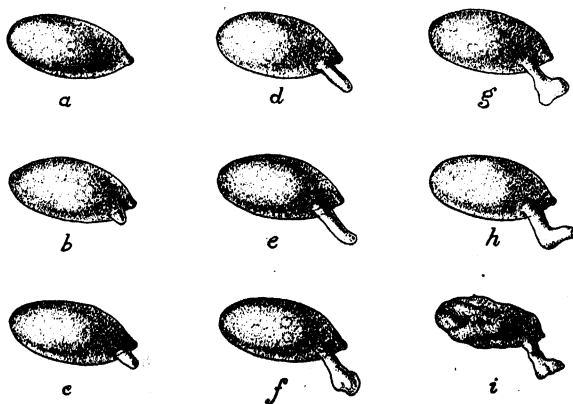


FIG. 4. *Erysiphe Polygoni*: drawings, the outlines of which were made with the aid of the camera lucida, showing the germination of a conidium on a cover slip suspended over concentrated sulphuric acid. Note that there is no apparent shrinkage of the spore until death occurs (i). $\times 500$.

tions on living material. Young mildewed leaves of *Polygonum* were folded on slides with the undisturbed conidiophores projecting beyond the edge of the folds. The conidiophores and conidia could be readily observed with both the low- and high-power objectives of the microscope. Continuous watching for 24 hr. showed that the conidia remain attached to the conidiophores unless disturbed by shock or a breath of air. Mildewed leaves were also suspended from the top of a covered glass dish and a slide was placed underneath the leaves. After two days, during which time the dish was undisturbed, no spores had settled on the slide. It would therefore appear that, in nature, mildew conidia are dislodged by air currents, rain, or other disturbances.

Hammarlund also stated that the conidia of *E. Polygoni* are sometimes produced in chains. The writers have studied this species on numerous hosts and have found only one mature conidium attached to each conidiophore. On one occasion, mildew was allowed to develop on bean leaves in the undisturbed and saturated atmosphere of a Wardian case. A few conidiophores were found in this material bearing chains of three or four conidia. The conditions in the Wardian case, however, were doubtless highly abnormal, and it seems probable that, in nature, not more than one conidium remains attached to each conidiophore in this species.

Structure of the Conidium

The Protoplast

The conidium of *E. Polygoni* is an ovoid colourless spore measuring 36μ by 17μ on the average. The first-formed conidium on a chain has a basal papilla only, each later-formed conidium is papillate at both ends. The protoplast presents a reticulate appearance and contains numerous globules that appear like vacuoles, these being from $1/5$ to $1/4$ the width of the spore. At the junctures of the granular cytoplasmic strands are very small globules of a highly refractive nature (Fig. 1f). Nuclei were not seen, but no attempt was made to stain them.

When living conidia were immersed in a 0.2 % solution of neutral red, their appearance was markedly different from that of conidia of several other fungi in the same stain. Conidia of *Fusarium culmorum* (W. G. Smith) Sacc., *Botrytis cinerea* Pers., and *Macrophoma* sp. were immersed in neutral red, and the stain immediately entered the spores and accumulated rapidly in typical vacuoles of various sizes; these made up the bulk of the spore contents. In contrast, the mildew conidia behaved very differently. Dead shrivelled conidia would absorb the stain instantly, the entire spore contents becoming red. Living turgid conidia at first did not absorb stain at all. Only after they have been allowed to stand in the stain from two to four hours did a small amount of stain penetrate the spore walls. By the time (two or three hours) germ tubes had become visible, a considerable amount of stain had entered the spores. The part of the conidium that first showed stain was always the papilla. It became pink, and then a faint coloration appeared in that

part of the protoplast nearest the papilla. After several hours, the stain spread through the spore. However, the larger globules which have the appearance of vacuoles did not absorb neutral red. By comparison with the stain-absorbing vacuoles of the other fungi mentioned, it was concluded that the large globules in the protoplast of *Erysiphe* conidia are not true vacuoles, or that they contain some material that has no affinity for neutral red. On the other hand, the stain accumulated in considerable concentration in the small refractive globules, which may, therefore, be true vacuoles or consist of some material having an affinity for neutral red (Fig. 1g).

The writers have concluded that the spore wall of the ungerminated mildew conidium is relatively impervious to water. As germination proceeds, the permeability of the papilla probably increases since stain and water appear to enter at the papilla end and to spread slowly from there. The large "vacuole" globules are possibly not of a watery nature as they do not absorb neutral red. The stain accumulated chiefly in the small refractive bodies, from which it may be deduced that they contain water. It will be shown later that the protoplast shrinks but slightly during plasmolysis, and it would seem justifiable to conclude that the mature conidium contains very little free water prior to germination.

The Cell Wall

The wall of the conidium is thin (1 to 1.5μ thick) except at the papilla end, smooth, and colourless. Sudan III accumulates slightly on the outer surface of spores immersed in it, but the wall itself is not stained. It is possible that there is a layer of some waxy material on the outside of the spore wall, although it must be admitted that the spores can be "wetted" very easily. Ungerminated turgid conidia do not take up neutral red, but, if fresh spores are washed with petroleum ether, allowed to dry for a moment, and then are flooded with neutral red, the stain at once colours them heavily. The waxy covering may be dissolved by the petroleum ether, rendering the spore wall permeable to neutral red.

No perforation in the wall is discernible at the papilla end when spores are examined with the oil immersion lens, and it is thought that no perforation is present. Rather, the papilla, despite the thickness of the wall at that point, represents a permeable area in a spore wall which is elsewhere relatively impervious to water.

Plasmolysis of the Conidia

It seemed possible that the tolerance of low humidity by the mildew conidia during their germination might in some way be correlated with a high osmotic pressure. Accordingly, an endeavour was made to determine the osmotic pressure of the mildew conidia by the plasmolytic method.

Plasmolysis in solutions of sucrose was first sought. To the writers' amazement, the spores remained unplasmolysed in the strongest concentrations of sucrose used. In fact, the conidia were able to germinate readily in a 5M solution of sucrose. A few fungi such as yeasts are able to germinate and

grow in honey (14), but the failure of mildew conidia to be plasmolysed by concentrated sucrose solutions is surprising. The reason is not, as yet, clear. It was noticed that the conidia shrank considerably in strong sucrose solutions, and it may be that the shrinkage of the spore as a whole masked the shrinkage of the protoplast.

Solutions of potassium nitrate were then employed, but plasmolysis was observed only at high concentrations. Due to some irregularities in the results, detailed report upon this phase of the work will be deferred until a later date. It may be stated here, however, that the osmotic pressure of the mildew conidia is very high and probably lies between 60 and 90 atmospheres. The plasmolysis observed was normal in that the protoplast always returned to its original volume when water was substituted for the plasmolyte. That the potassium nitrate was not toxic to the conidia is shown by their ability to germinate in concentrations of that salt up to the strength inducing plasmolysis. The shrinkage of the protoplast was slight in all instances and was slow in taking place, presumably owing to the impervious nature of the conidium wall necessitating the passage of liquids into the conidium via the papilla. Even at high concentrations of the plasmolyte, the protoplast did not assume a spherical shape nor undergo marked reduction in volume. This is interpreted as additional evidence that the protoplast contains very little water that could be withdrawn during plasmolysis.

Whether or not high osmotic pressure is an aid to mildew conidia in taking up water is difficult to say. Since it will be shown in this paper that the conidia of *E. Polygoni* are capable of germinating well in a current of air dried by passage through concentrated sulphuric acid, it would appear that they are independent, in respect to germination, of their ability to take up water. Further, the question of taking up water during germination may be one of imbibition rather than osmotic pressure.

Germination of Conidia

The conidia of *E. Polygoni* were found to germinate, on the average, in one hour and 45 min., the minimum time recorded being one hour and 15 min. The germ tube emerges slightly to one side of the end of the conidium and, in moist air, is generally straight and terete. In dry air, it may be short and often convoluted (Fig. 5c). Only one germ tube was found to be produced by each spore.

The writers have heard the criticism made of Yarwood's work that the germ tubes observed by him (produced under conditions of low humidity) might be abnormal swellings on the spores. This idea is entirely erroneous, for the writers have observed the development of thousands of normal germ tubes on spores kept in extremely dry air.

Conidia of various mildew species were subjected to germination tests under conditions of humidity ranging from zero (approximately) to 100%, according to the procedure outlined above. The temperature during these experiments

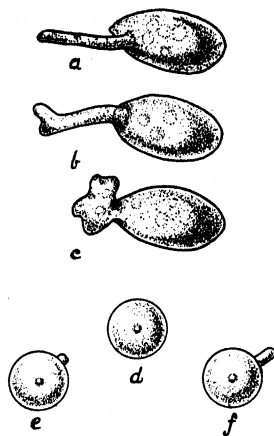


FIG. 5. *Erysiphe Polygoni*: a, b, c, variation in form of germ tube at different humidities, a and b in moist air, c, in very dry air; d, e, f, drawings made with the aid of the camera lucida showing a conidium germinating on a cover slip suspended over concentrated sulphuric acid and demonstrating that the conidium does not change in outline during germination when seen from above. $\times 500$.

varied but slightly from 22° C. although no attempt was made to control it. The results of these experiments are given in Table I.

The results fully uphold Yarwood's contentions, for it will be seen from Table I that (except for the two varieties of *S. Humuli*) the mildews examined germinated as well at low humidity as at high. In no experiment was there a

TABLE I

PERCENTAGE GERMINATION OF POWDERY MILDEW CONIDIA UNDER DIFFERENT CONDITIONS OF HUMIDITY AT 22° C.

Organism	Relative humidity in germination chamber, % ¹											
	0	2.5	4.5	7.5	10.5	20	32.5	54.7	79.5	90	95	100
<i>Erysiphe Polygoni</i>	36	46	20	24	27	21	31	24	41	43	52	12
from <i>Delphinium</i>	21	16	—	18	28	18	37	34	26	29	27	31
	23	23	—	21	39	39	38	31	32	36	29	29
<i>Erysiphe Polygoni</i>	33	6	1	4	28	16	2	—	2	4	0	7
from <i>Polygonum</i>												
<i>Sphaerotheca Humuli</i>	0	0	0	0	0	0	4	—	0?	11	6	14
from rose												
<i>Sphaerotheca Humuli</i> var.	0	0	0	0	0	0	6	—	0?	22	14	24
<i>fuliginea</i> from pansy												
<i>Erysiphe graminis</i>	22	16	14	11	15	31	11	—	20	13	12	10
from <i>Poa pratensis</i>												

¹ Germination percentages given only as nearest whole numbers.

clear-cut optimum humidity, and the writers therefore agree with Yarwood in concluding that mildew conidia are tolerant of a wide range of humidity as regards germination or, to put it another way, are independent of humidity.

Neither of the two varieties of *S. Humuli* tolerates conditions of very low humidity, no germination being observed below 32.5% and but little below 90%. However, as compared with the requirements of most fungi, 32.5% is remarkably low. Neither the rose nor the pansy mildew is generally found in hot dry locations, and it is not surprising that they should be less tolerant of low humidity than the other kinds tested, all of which may be found in open, dry situations.

In the above experiments, a possible source of error was considered. It was thought that some time might elapse before conditions of low humidity were really established in the germination chambers. Thus, if the laboratory air in the summer had a relative humidity of, say, 50% at the time of the test, the conidia placed in the closed chamber would begin their germination at that humidity and might be subjected to low humidity only after the regulating solution had withdrawn moisture from the air of the germination chamber. And since the conidia germinate in a very short time, this lag in the establishment of low humidity might lead to serious error. In order to leave no possibility of doubt regarding the ability of the conidia to germinate in extremely dry air, the following experiment was carried out.

Freshly gathered conidia on cover slips were placed in a glass bottle provided with a ground glass stopper and two tube outlets. One of the outlets was connected to a series of three tall wash bottles and these were filled with concentrated sulphuric acid. Air was drawn through the whole apparatus immediately after the spores were placed in the germination chamber. The air, bubbling very slowly through the wash bottles, must have been very desiccated when it reached the conidia. The air originally present in the germination chamber was, in this way, removed at once, and one could be certain that the conidia had been subjected to dry air from the beginning of the test. Four such tests were made for which the average germination percentage was 35.5%. The average for spores allowed to germinate in the laboratory air at the same time was 52%. This experiment indicates that there is no doubt about the ability of mildew conidia to germinate in dried air.

Constancy of Volume of Conidia during Germination

Yarwood (23) stated that he had observed a shrinkage of as much as 24% in volume of turgid conidia when these were germinating in an atmosphere of 80% relative humidity. The writers made a special effort to obtain confirmation of this finding, with negative results. It had been found that excellent germination may be obtained when the conidia are merely caused to fall on dry slides and allowed to germinate in the air of the laboratory. Conidia of *E. Polygoni* from *Delphinium* and from *Polygonum aviculare* were dusted on dry slides and then placed on the stage of the microscope. Camera lucida drawings were made of several conidia under the high power at inter-

vals of about 20 min. (Fig. 4). In another series of observations, measurements were taken at intervals with an ocular micrometer. During the experiments, which were made on different days, the air of the laboratory ranged from 65 to 80% relative humidity.

Up to three hours, within which time the conidia had germinated, the writers failed to observe any change in volume of the dozens of conidia observed continuously. It was suggested to the writers by Professor A. H. R. Buller of the University of Manitoba, that depression of the conidia along the short axis might take place without resulting in change of outline seen from above the spore, when the spore lay with its long axis parallel to the glass slide. However, a few spores were found that had landed on one end, and several of these were watched carefully during germination. Here too, no change in outline could be observed (Fig. 5-d, e, f). Tests were also made at zero relative humidity with a similar result.

The writers are forced to conclude that, in their material at least, there was no change in volume of the spores during germination. Yarwood's measurements, from which he deduced that shrinkage takes place, were made after 5, 10, and 24 hr. The writers have rarely found that germinated conidia remain alive on slides for more than five hours. After about four hours, growth ceases (Fig. 4h), and one observes a marked change in the appearance of the protoplast which becomes darker and denser. At about this time a marked wrinkling of the spore is evident and the whole conidium shrinks (Fig. 4i). It seems certain that the conidium is dead, however, before the shrinkage occurs.

Non-Germination of Mildew Spores *in situ*

Having established the fact that the spores of some powdery mildews are independent of moisture for germination, the question naturally arises: are the conidia capable of germinating *in situ*? In nature, mildew conidia produced on the conidiophores above the surface of the leaf might be expected to germinate as soon as they are produced.

Examination of the illustrations of such careful observers as the brothers Tulasne (20) fails to reveal any spores germinating while still attached to the conidiophores.

Several times the writers kept material of *E. Polygoni* under constant observation for a period of 24 hr. The mildewed leaves were folded so that the conidiophores projected into the air and could be readily examined under low power. As a result of these observations, it may be stated that the conidia do not germinate as long as they remain attached to the conidiophores. But one has only to shake the conidia on to a dry slide to see them germinate, and it is necessary to offer some explanation as to why they remain ungerminated while attached to the conidiophores.

At first it was believed that, as long as the conidia remain attached, some chemical substance on or in the conidiophore might inhibit germination. To

test this hypothesis, a large quantity of mildew mycelium was carefully scraped from infected leaves and added to a small quantity of sterile water, and thoroughly macerated. Fresh conidia were then suspended in the water and macerated mycelium. As control experiments, conidia were suspended in sterile distilled water and in water containing macerated host tissue. Mildew conidia do not germinate well in water, but 15% germination was obtained in the sterile water and in host tissue. The same amount of germination was obtained in the macerated mycelium, indicating strongly that there is no chemical substance present in the mycelium to inhibit the germination of the conidia.

Next, the matter of maturity was considered. It has been shown that the conidia become disjoined from the conidiophores by shock or wind and it seemed possible that the conidia might be immature until just before they are able to be dislodged. If this were so, conidia artificially detached prior to maturity could not germinate when detached.

A mildewed leaf was held firmly with forceps and then shaken violently in order to dislodge all conidia possible. The leaf was then examined under the low power of the microscope and several conidiophores bearing firmly attached conidia were found. By means of careful micromanipulation, these immature conidia were removed. They were dislodged only after considerable probing with the needles of the manipulator. Despite the fact that not 1 of the 10 conidia so obtained was sufficiently mature to drop from its conidiophore, 9 of the 10 germinated after being detached. It would appear from this observation that, provided the formation of the conidium is complete, it can germinate, whether ready to drop off of its own accord or whether artificially dislodged. The suggestion that the conidia do not germinate *in situ* because they are undergoing a process of maturation is not borne out by this observation.

Relation of Carbon Dioxide to Germination

In considering the conditions within the mature conidium that might be responsible for its failure to germinate as soon as it is produced, it may be well here to restate certain facts regarding its structure. The septum which abstricts the terminal conidium is, at first, perforate, providing for cytoplasmic connection with the conidiophore and mycelium below (Fig. 1c). Later, the septum becomes entirely closed and the conidium may be supposed to be separated physiologically from the conidiophore. After separation, the continued respiration within the conidium might do two things: (1) increase the internal concentration of carbon dioxide and, at the same time, (2) decrease the internal concentration of oxygen. It has already been shown in this paper that the conidium wall is relatively impervious to water except at the papillate end. If it be assumed that it is also relatively impervious to gases, then, as long as the conidium remains attached to the conidiophore, either the accumulation of carbon dioxide within the mature conidium or the low concentration of oxygen might prevent its germination. The conidium appears

so firmly attached to the conidiophore that it seems safe to assume that the permeable papilla would not be exposed to the outside air until after the conidium had become detached.

To test this theory, mildew conidia were first subjected to an atmosphere of air containing excess of carbon dioxide. Freshly dislodged conidia collected on glass cover slips, were placed in a large glass-stoppered wash bottle provided with two exit tubes. Air was drawn into the chamber and a small proportion of carbon dioxide (approximately 1 part in 10) was allowed to mix with the air entering the germination chamber. The gas mixture was allowed to pass slowly through the germination chamber for 30 min. Ground-glass cocks on either side of the germination chamber were then closed and the spores allowed to remain in the gas mixture. A control experiment was set up using a duplicate apparatus lacking the arrangement for adding carbon dioxide.

After two hours, excellent germination had taken place in the control apparatus, while in the presence of added carbon dioxide no spores had germinated. The ungerminated spores were then removed from the germination chamber in which there was added carbon dioxide and they were allowed to stand exposed on the laboratory table. Two hours later, these spores were found to have germinated. The results of the experiment are presented in the following table.

TABLE II

EFFECT OF CARBON DIOXIDE ON GERMINATION OF MILDEW CONIDIA

Trial No.	Percentage germination		
	In chamber with added CO ₂	In chamber with air	Same conidia shown in Column 2, two hours after removal from CO ₂ chamber
1	0	45	43
2	0	50	55
3	0	50	44

From these results it may be concluded: (1) that carbon dioxide in concentration of approximately 10% by volume prevents mildew conidia from germinating during the two hours required for germination of conidia exposed to atmospheric air; and (2) that conidia that have been held in carbon dioxide for two hours germinate as well after they have been removed from the carbon dioxide as though they had not been subjected to experiment.

The writers hoped to be able to extend the above experiment to find the critical concentration above which no germination could occur. However, much difficulty was experienced at first in obtaining consistent results in the experiment. This proved to be due to the extreme toxicity to the spores of vulcanized rubber used in the form of rubber tubing and stoppers in the apparatus. The source of trouble was not located until late in the work

and it was not possible to put the experiment on a more quantitative basis. However, the results are so clear-cut as to seem convincing, even in their unrefined form.

Relation of Oxygen to Germination

Experiments were carried out to determine whether or not the conidia of *E. Polygoni* are capable of germinating in an atmosphere in which the proportion of oxygen is less than that present in ordinary air. At first an attempt was made to remove oxygen from atmospheric air by passing the latter very slowly through three wash bottles containing strong alkaline pyrogallol. The conidia were placed in the germinating chamber as in the previous experiments, and a control apparatus was set up in which washed air passed directly over the conidia without first passing through pyrogallol. The results of several tests were entirely negative; about 65% germination was obtained in the air which had passed through pyrogallol as well as in the control apparatus.

The conclusion to be drawn is either that the conidia are capable of germinating in oxygen-free air or that the pyrogallol did not remove sufficient oxygen to cause any checking of germination. The latter explanation seemed the more probable.

A further experiment was conducted in which a heated combustion tube was connected between the pyrogallol wash bottles and the germination chamber. Into the combustion tube was inserted a roll of freshly reduced copper gauze which was heated as the air from the intake was drawn slowly over it. Again negative results were obtained.

Conidia were then placed in an atmosphere of "tank" nitrogen. The gas was allowed to pass through the apparatus for 20 min. only, to sweep out the air, and pinch cocks were then closed on either side of the germination chamber. In the control experiment the conidia were subjected to ordinary air in a duplicate apparatus. This time, positive results were obtained as shown in Table III.

TABLE III
EFFECT OF NITROGEN ON THE GERMINATION OF MILDEW CONIDIA

Trial No.	Percentage germination		
	In chamber with nitrogen	In control chamber	Same conidia shown in Column 2, two hours after removal from nitrogen chamber
1	0	12	10
2	2	80	80
3	2	75	75
4	0	27	18
5	6	80	80
6	0	27	30
7	3	80	80

It will be seen that in all trials, germination was prevented or markedly checked when the conidia were held in nitrogen. In trials Nos. 1, 4, and 6, no germination had occurred in two hours in nitrogen, whereas, in the controls, 12, 27, and 27% germination, respectively, was observed. In all trials, the germination of conidia held for two hours in nitrogen was approximately the same after the conidia had been removed from nitrogen and allowed to germinate on the laboratory table as though they had not been subjected to experiment.

The small percentages of germination in nitrogen observed in trials Nos. 2, 3, 5, and 7, may be explained by the fact that "tank" nitrogen contains as high as 4% oxygen and this, presumably, is sufficient to allow a small proportion of spores to germinate.

From these experiments it may be deduced (1) that lack of oxygen or a very low concentration of oxygen prevents germination of the mildew conidia and (2) that, at least for a period of two hours, conidia are not permanently injured by lack of oxygen as shown by the fact that they germinate well when oxygen is provided.

To date, the writers have not determined the critical concentration of oxygen below which no germination can take place. This would have to be carried out using chemically pure nitrogen to which could be added as much oxygen as desired.

Toxicity of Vulcanized Rubber to Mildew Conidia

During the course of the above experiments, considerable difficulty was experienced at first in obtaining any germination within the apparatus used for examining the effect of gases on germination. Finally it was found that both ordinary rubber tubing and rubber stoppers are extremely toxic to mildew conidia. When ground-glass connections and pure gum-rubber tubing were used in the apparatus, excellent germination within the apparatus was obtained. The high degree of toxicity of vulcanized rubber seemed to warrant some actual tests, and the following experiments were therefore performed.

Spores were suspended on dry cover slips over Van Tieghem cells. In each cell was placed a small fragment of rubber stopper (about 2 mm. in diameter). Twenty-four cells were arranged along with a series of the same number of controls not containing rubber fragments and a series of cells containing fragments of pure gum rubber. The results of this test are given in Table IV.

This experiment shows that some substance in vulcanized rubber is highly toxic to the spores of *E. Polygoni*. Since non-vulcanized rubber is not toxic and vulcanized rubber contains sulphur, and also since it is a matter of common knowledge that sulphur is a good fungicide for the control of diseases caused by powdery mildews, it would appear probable that the substance present in rubber that is toxic to the mildew spores is a compound of sulphur. The conidia failed to germinate when they had been removed from the cham-

TABLE IV
EFFECT OF VULCANIZED RUBBER ON GERMINATION OF MILDEW CONIDIA

Percentage germination after two hours ¹		
In precence of vulcanized rubber	In presence of non-vulcanized rubber	In control
3.5	37	41 57

¹ Average of 24 counts of 100 conidia each.

bers containing vulcanized rubber, which shows that they had been injured permanently. A search for the toxic principle of vulcanized rubber might lead to the discovery of an excellent means of control of powdery mildews¹.

Discussion

Unlike the spores of all other fungi, so far as is known, the conidia of certain of the Erysiphaceae are capable of germinating under conditions of extremely low humidity. The writers agree with Yarwood (23) in concluding, not that low humidity is essential to the luxuriant development of mildew, but rather that, unlike other fungi, the powdery mildews are capable of developing over a wide range of relative humidity and that they are well adapted to dry atmospheric conditions. This adaptation appears to be related to the physiological and morphological peculiarities of the conidia.

The conidium, as it matures, becomes walled off from the conidiophore by a septum but remains firmly attached to the conidiophore, contact being maintained between a papilla at the proximal end of the conidium and a papilla at the distal end of the conidiophore. The wall of the mature conidium appears to be relatively impervious to water and probably to gases except at the papillate end through which stain has been shown to enter.

After maturation, the conidium remains on the conidiophore unless detached by shock, wind, etc., and in this position it is sealed, if not hermetically, at least sufficiently to prevent the ingress of oxygen from the outside or release of carbon dioxide from the inside to the extent necessary to initiate the germination process. Thus it fails to germinate *in situ*, even though the relative humidity of the air surrounding the spore may be suitable for germination.

Upon being dislodged from the conidiophore, the seal formed by the firm contact of the papilla with the conidiophore is broken. The end of the papilla is then exposed to the surrounding air and, being more permeable than the remainder of the conidium wall, it allows carbon dioxide to pass out from the protoplast and oxygen to pass in.

¹ Since these statements were written, the senior author has carried out experiments which show that as little as 0.01 gm. of vulcanized rubber per 100 cc. of air will prevent mildew spores from germinating on dry slides in Petri plates.

The writers have not been able to prove, up to the present, whether low oxygen concentration or high carbon dioxide concentration is responsible for checking germination, but they are inclined to the belief that it is the accumulation of carbon dioxide within the protoplast that is of greater importance in this regard.

When Yarwood's work was first published, the criticism was raised (but apparently not published) that the increase in volume that would seem bound to result from the spore producing a germ tube, must come from the uptake of water, and the problem arose as to how the spore could withdraw water from dry air. It is suggested by the writers' preliminary experiments that the conidium of *E. Polygoni* has a very high osmotic pressure, which might account for the ability of the conidium to withdraw water from moderately dry air. This mechanism does not seem, however, to account for the germination of spores in a current of air dried by passage through sulphuric acid.

The protoplast of the conidium appears quite unlike that of the spores of *Fusarium culmorum*, *Botrytis cinerea*, and *Macrophoma* sp. with which it has been compared. In those fungi there are large watery vacuoles which readily absorb neutral red, while no such vacuoles appear in the ungerminated conidium of *E. Polygoni*. There is probably very little water present in the mildew conidium, the protoplast consisting of a gel-like material.

Upon the release of carbon dioxide from the conidium, respiration would begin if sufficient oxygen were present. Conceivably, as a result, the viscid protoplast would be converted into materials more labile and voluminous, and the increase in volume necessary for the production of a germ tube might come from this source alone.

It has been stated that neutral red enters living conidia, at first extremely slowly, and then more rapidly as germination proceeds. Probably the sporelings do require water after a time; and, under natural conditions, they might be in a position to absorb it. At low humidity, on dry slides, the germ tubes seldom become as long as the spores and they soon die. This may well be due to the lack of water necessary for further development.

Quite regularly, many conidia shrivel early in germination tests at low humidity. One may ask: why, under dry conditions, do some conidia germinate while others shrivel and die? One explanation that might be offered is that, for some reason, in the conidia that shrivel, the papilla does not function normally. It has been noted that dead conidia absorb neutral red immediately upon immersion in it, which would indicate that the papilla end of the spore becomes entirely permeable when death of the spore takes place. Possibly, in some spores, the papilla is too permeable and, under dry conditions, it may allow the contents of the spore to suffer through drying. The writers hope to throw more light on this and other problems by means of further research now in progress.

Finally, the writers suggest that, at this stage of development of the present problem, caution should be observed in applying the ideas brought forth in this paper to questions of the epidemiology of mildew on crop plants.

There seems no doubt that certain mildew species tolerate extremely low humidity. However, infection (involving the establishment of the mycelium on the host and haustoria within the host cells) may well reach its optimum under conditions different from those which bring about optimum germination. The writers' experience with *E. Polygoni* leads them to believe that higher humidity may be necessary for infection than is necessary for germination. Further research along this line is needed to clarify the situation.

Acknowledgments

The writers are indebted to Professor A. H. R. Buller, Professor Emeritus of the Department of Botany of the University of Manitoba, who, from time to time, critically discussed the work with the senior author and made valuable suggestions regarding the preparation of the manuscript. Professor William Leach, Professor of Botany at the University of Manitoba, also gave the writers the benefit of his criticism. It is a pleasure to acknowledge the invaluable aid given by these gentlemen.

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STUDIES ON FILM-FORMING YEASTS

II. FILM-FORMING YEASTS IN RENNET BRINE¹

By V. E. GRAHAM² AND E. G. HASTINGS³

Abstract

In the commercial production of rennet extract, calves' stomachs are soaked in brine tanks held at a low temperature. Unless special precautions are taken, a heavy scum forms on these tanks. Salt tolerant yeasts of the genus *Debaryomyces*, which grow well at low temperatures, are chiefly responsible for this scum from which *D. tyrocola*, originally isolated from cheese, and *D. Guilliermondi*, originally isolated from sausages, were isolated. Attempts to isolate these species from the contents of a calf's stomach, salted calves' stomachs, dried calves' stomachs, and soil were unsuccessful. These species did not grow in a medium containing 20% sodium chloride, nor in one in which the pH had been lowered to 2.0.

Introduction

Commercial rennet extract is made by removing rennin from the fourth stomach of a young calf, the extraction being accomplished by prolonged soaking in a brine solution. Some protein and other constituents are removed from the stomachs in this process and the resulting mixture would undoubtedly spoil quickly were it not for the preservative action of the salt and the low temperature maintained in the extracting rooms. If the material in the extracting vats is allowed to remain at rest a grey scum frequently forms on the surface. Vigorous agitation suppresses scum formation. The nature and significance of this scum has not been understood but its presence has been a matter of some concern.

Yeasts Isolated from Rennet Brine

Several samples of scum were obtained and studied by the methods already described (4). Microscopic observation showed that small, round to oval yeasts were present. Inoculation of enrichment medium No. 1 with this material resulted in the formation of a typical scum when the medium was incubated at room temperature. Six strains of film-forming yeasts were isolated from the enrichment cultures by plating on acid glucose yeast extract agar. The pH of this medium was adjusted to 4.5 with lactic acid.

All these cultures, designated R_1 , R_2 , R_3 , R_4 , R_{19} , and R_{22} , were found to belong to the genus *Debaryomyces* Klockner. They have been described

¹ Manuscript received in original form June 2, 1941, and as revised, October 20, 1941.

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in detail elsewhere (3). Stelling-Dekker (9) characterized the genus as follows: "The cells are predominantly round but may also be oval, and are usually small. Vegetative reproduction takes place by multilateral budding. In wort a sediment is formed, usually also a ring, and many species also form a film. Asci are produced after isogamic or heterogamic copulation or parthenogenically. The spores are round, mostly with an oil drop in the centre. The spore wall is warty, but this is often difficult to observe. In nearly all cases there is only one spore per ascus. Usually there is no fermentation; however, one species has a good fermentation. Nitrate assimilation is negative. On a medium containing ethyl alcohol there is good growth often with the formation of a thin film." The cultures examined in the present study agree with this description except that the results on ethyl alcohol utilization were inconclusive.

Regarding the difficulty in observing projections on the spore wall in this genus, Mrak and Bonar (7) have shown that the ease with which these may be seen is determined chiefly by the size of the spore in relation to the size of the ascus. When cultures are grown at temperatures of 22° C. or higher there is a tendency for the spore to fill the ascus, but when incubated at considerably lower temperatures the spore is smaller in relation to the size of the ascus and the markings are more distinct. In the study reported here, experience with cultures grown at room temperature and in the refrigerator confirms this observation.

Cultures R_1 , R_3 , and R_4 , were identified as *Debaryomyces tyrocola* Konokotina. This species was isolated by Konokotina (5) from "Hollandish" cheese. It is interesting that this organism has now been found in rennet brine. Konokotina isolated four strains of which Stelling-Dekker has described three. The differentiation of the strains is based chiefly on the size of the cells. The spherical cells of Cultures R_1 , R_3 , and R_4 , varied in diameter from 2.4 to 3.5 μ and therefore belong to Strain III. These three cultures formed only light films and are probably only partly responsible for the scum found on the rennet extraction brine.

Cultures R_2 , R_{19} , and R_{22} , are probably strains of *Debaryomyces Guilliermondi* Dekker. Stelling-Dekker described two strains of this species, viz., *Fm* and *K*. The cultures referred to correspond closely to her description of Strain *K*. According to Stelling-Dekker this strain liquefies gelatine, sporulates freely on Gorodkova agar, produces a white, dry, wrinkled film on wort and a "brown-yellow-grey" colour on old wort agar slopes. The development of a pink to light brown colour, particularly when grown at 12° C., was quite marked and differentiated these cultures from all others. This colour was most pronounced on wort agar. It did not develop until the cultures had been incubated for two to three weeks. Cultures R_{19} and R_{22} are almost identical. Culture R_2 is different in some respects and, while it is tentatively classified as a strain of *D. Guilliermondi*, further study may show that it is a new species of this genus. The film formed by these three cultures is white,

with a dry 'mealy' appearance. Undoubtedly this species is responsible for most of the scum on rennet extraction brine.

Both species of *Debaryomyces* isolated in this study grew well in a suitable liquid medium containing 15% of sodium chloride but a 20% solution inhibited growth completely. They did not grow at 37° C. but grew luxuriantly at 12° and 24° C. It is apparent, therefore, that if other conditions are satisfactory, they would develop readily at the temperature and salt concentration used in rennet extraction. Mrak and Bonar (8) reported the isolation from pickle brine of strains of *D. membranaefaciens* Naganishi and *D. Guilliermondii* which grew in a 24% solution of sodium chloride.

Influence of Hydrogen Ion Concentration on Growth of Strains of *Debaryomyces*

A medium containing 0.5% peptone, 1% sodium chloride, and 1% glucose was prepared. The pH of different lots of this medium was altered by the addition of hydrochloric acid or sodium hydroxide. The pH of each lot was determined colorimetrically after sterilization. These media were inoculated with the six strains of *Debaryomyces* isolated from rennet brine and also with the four stock cultures recorded in the table. The relative amount of growth after 10 days' incubation at room temperature is indicated in Table I by the

TABLE I
INFLUENCE OF pH ON GROWTH OF STRAINS OF *Debaryomyces*

Culture	pH of medium					
	2.0	2.5	3.8	4.8	6.8	8.0
<i>R₁</i>	Nil	Nil	++	++	++	+
<i>R₂</i>	Nil	Nil	++	++	++	+
<i>R₃</i>	Nil	±	++	++	++	±
<i>R₄</i>	Nil	±	++	++	++	±
<i>R₁₉</i>	Nil	Nil	+++	+++	+++	+++
<i>R₂₂</i>	Nil	±	+++	+++	+++	+++
<i>D. membranaefaciens</i>	Nil	±	++	++	++	±
<i>D. membranaefaciens</i> var. <i>hollandicus</i>	Nil	+	++	++	++	++
<i>D. Guilliermondii</i>	Nil	Nil	++	++	++	++
<i>D. Guilliermondii</i> var. <i>Nova Zeelandicus</i>	Nil	Nil	+	++	++	++

NOTE: (+++) = Vigorous growth.
 (++) = Good growth.
 (+) = Growth light but definite.
 (±) = Growth doubtful (islands only).

conventional symbols. These results show that the growth of all strains was inhibited at a pH of 2.0. At pH 2.5 five strains were completely inhibited and four others developed only small islands of growth. *D. membranaefaciens* var. *hollandicus* Lodder grew slowly at pH 2.5 but this species was not found in the samples of rennet brine examined.

Liquefaction of Gelatine

Stelling-Dekker reported that *D. Guilliermondi* liquefied gelatine in 60 days. Culture R_2 , when freshly isolated, liquefied gelatine in 45 days, but when tested a year later it did not cause liquefaction in nine weeks. Cultures R_{19} and R_{22} did not liquefy gelatine in nine weeks. Likewise, stock cultures of *D. Guilliermondi* and *D. Guilliermondi* var. *Nova Zealandicus* Lodder* did not liquefy gelatine in nine weeks. It is doubtful whether liquefaction, when long delayed, is of importance in classifying these species of *Debaryomyces*.

The Source of Film-Forming Yeasts in Rennet

The species of *Debaryomyces* found in rennet extraction brine did not grow at 37° C., and, therefore, one would not expect to find them as natural inhabitants of a calf's stomach. The careful examination of the contents of the third and fourth stomachs of a young calf, by the enrichment procedure, revealed no *Debaryomyces* whatever. Likewise, the examination of salted calves' stomachs and dried (blown) calves' stomachs purchased by a rennet manufacturer revealed no yeasts of this genus. Samples of soil taken to a depth of 2 in. from lawn sod, from a vegetable garden, and from the shore of Lake Mendota, Wis., were also examined but yielded no *Debaryomyces*. Both of the species found in rennet extracting brine were isolated by Césari and Guilliermond (2) from sausages in which these species were responsible for white patches on the casings. They reported that several species of *Debaryomyces* were present on salted meats but that they were not present in salt itself. Mrak and Bonar (8) found strains of *Debaryomyces membranaefaciens* in pickle brine.

A strain of *Debaryomyces tyroloca* Linn. was isolated from the larvae of *Lymantria monacha* Linn. by Batschinsky (1). Kouokotina [sic] and Krassihukow (6) reported the isolation of yeasts closely resembling *Debaryomyces tyroloca* from Turkestan grapes, the pericarp of cucumbers, birch sap, beef hides, the larvae of *Agrotis segetum* Schiff., the intestine of a porpoise, the excrement of a fox, a honey comb, and rancid butter. It is evident from these reports that species of *Debaryomyces* may be found on many different materials but the natural source of the contamination of rennet brine is still unknown.

* Kindly supplied by Dr. E. M. Mrak, Fruit Products Laboratory, University of California, Berkeley, Calif., U.S.A.

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PEAT AND COMMERCIAL FERTILIZERS AS AMENDMENTS FOR GRAY WOODED SOILS¹

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Abstract

The nitrates in pot cultures of gray wooded soil were generally affected by the various amendments as follows: increased by applications of neutral or acid subsurface peat and not depressed by neutral or acid surface peat; increased by ammonium phosphate alone and in combination with the surface and subsurface peats, and by ammonium sulphate in combination with the subsurface peats, but slightly depressed by triple superphosphate in combination with the subsurface peats.

The wheat crop yields in pot cultures of gray wooded soil were generally affected by the various amendments as follows: increased somewhat by peat alone, and especially by subsurface peat; increased substantially by ammonium phosphate alone, but increased to a greater extent in all three series by a combination of ammonium phosphate and subsurface peat; increased substantially by ammonium sulphate and subsurface peat, but not by triple superphosphate and peat.

The plate counts of bacteria in treated cultures of gray wooded soil increased for two to four months, and then decreased. The ammonium phosphate plus subsurface peat treatment gave the highest counts, the ammonium phosphate plus surface peat the second highest, and the ammonium sulphate plus subsurface peat the third highest. The differences between the effects of triple superphosphate plus subsurface peat, ammonium phosphate, surface peat, and subsurface peat, were small. The control gave only a small increase during the course of the experiment.

The ratio method showed that there are very large numbers of bacteria in gray wooded soil. Instead of ranging from about 500,000 to about 8,000,000 per gm. of soil as with the plate count method, the numbers varied from about 200,000,000 to 2,000,000,000. The numbers reached a maximum after three months' incubation. The ammonium phosphate plus subsurface peat treatment again gave the highest average number, and the succeeding order was also about the same as in the case of the plate counts.

When mixed with gray wooded soil, surface peat, though highly carbonaceous, did not cause any significant decrease in nitrate at first, and produced some increase later. Straw caused some decrease at first, but the later recovery counterbalanced this loss. Cotton caused a decrease to practically none for about three months, and even when combined with ammonium phosphate caused some decrease. Ammonium phosphate alone and in combination with straw and surface peat produced large increases in nitrate. In a duplicate experiment with black soil the trends were similar, but the nitrate was not reduced to the same extent by straw and cotton, and when ammonium phosphate was added with the organic matter, nitrate was not reduced by peat, straw, or cotton.

Introduction

The gray wooded soil zone of central-western and northern Alberta is the largest of the main soil zones of this province. Gray wooded soils are commonly badly leached, light in colour, poor in fertility, and slightly acid in reaction. They especially lack organic matter and often bake badly when dried. When plowed the field has a light coloured, ashy appearance. These soils are also very deficient in nitrogen and sulphur and somewhat deficient in phosphorus

¹ Manuscript received September 19, 1941.

Contribution from the Department of Soils, University of Alberta, Edmonton, Alta., with financial assistance from the Carnegie Research Grants Committee of the University of Alberta.

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and potassium. Consequently they commonly do not produce satisfactory crops, and, as the gray wooded soil districts are gradually being settled, the problem of applying fertilizers to increase yields is becoming very important.

A number of reports of investigations concerning Alberta's gray wooded soils have been published (9, 16, 17, 18, 19, 20). These soils are podsollic in nature, but differ to some extent from the podsollic soils of Eastern Canada and the United States. They are less acid and commonly contain a lime layer in the *B* horizon below the leached layer.

Interspersed among these gray wooded soils are many large and small areas of peat or "muskeg" swamps. These peats are commonly classified as "high moor" or "moss" peats and it has been estimated that they cover possibly as much as 25,000,000 acres in Alberta. The vegetation of Alberta peats has been studied and described by members of the Department of Botany of the University of Alberta (6). The surface layer of these "muskegs" is commonly light in colour, and but slightly decomposed. The underlying layers are darker in colour and decomposed to a greater degree. Analyses of Alberta peats have shown that they usually contain high percentages of cellulose in the surface layers; they are quite variable in nitrogen and phosphorus content, commonly low in potassium and high in sulphur (8, 10, 15, 16).

Experiments with peat as an amendment for gray wooded soil were suggested by the fact that these soils are often very deficient in organic matter, and also by the fact that commercial fertilizers had proved highly beneficial to wheat when applied after clover had been plowed down, in experiments at Breton, Alberta (18). The experiments are particularly apt because in many parts of the gray wooded soil zone the peat swamps are so interspersed that nearly every farmer is within a few miles of such a swamp if he does not actually have one on his own farm. Consequently this investigation was started with the purpose of finding out whether the application of such peats would benefit the gray soils. It was felt that the benefits could be both physical and chemical, but it is mainly with the chemical effects that this investigation is concerned.

Methods

The investigation was carried out mainly with pot and tumbler soil cultures and, in general, the results are based on three determinations. First, wheat crops were grown in pots and yields taken as an indication of whether the different treatments of the gray wooded soil are beneficial or otherwise. Secondly, nitrates in pot cultures were determined at regular intervals as an indication of the rate at which the peat and nitrogenous fertilizer incorporated with the soil are nitrified or made available to plants. Thirdly, the effect of peat and commercial fertilizers on microbiological activity in tumbler cultures of gray wooded soil was measured by making bacterial counts periodically. Also, a comparison was made of the effect of peat and some other cellulosic materials (straw, cotton), in pot cultures, on nitrification of gray soil.

The experiment for the determination of nitrates was done in conjunction with that for crop yields and was laid out as follows.

Gray wooded soil (surface seven inches) from Fallis was sifted through a coarse sieve, mixed uniformly, and weighed into 1-gal., 6-in. glazed pots. Stony Plain high lime peat was used in two experiments and Chip Lake acid peat in another experiment. The coarsely ground peat was mixed uniformly with the soil at the rate of three tons of dry peat per acre (or per 2,000,000 lb. of soil).

The treatments are shown in Table I. There were four replicate pots of each treatment. Three of these were used to grow wheat crops and the fourth in each case was uncropped and kept for the determination of nitrate nitrogen. Reward wheat was sown at the time of applying fertilizers. The pots were watered with distilled water. As far as possible equal numbers of plants were left to develop in each pot and the number was 16. Shortly before each successive crop was planted the soil was emptied out, mixed, and returned to the same crock. About one to two months' time elapsed as a rule between the time of harvesting the crop and replanting; this is indicated in the yield tables (III, V, and VII).

The yields of wheat were obtained by cutting the crop close to the soil and drying thoroughly before weighing and threshing.

Representative composite samples of soil for nitrate determinations were obtained by taking samples from each pot in five different places to the depth of the pot, with a small auger.

The nitrate determination was carried out by the phenoldisulphonic acid method as modified by Harper (5).

In determining bacterial numbers two methods were used, the cultural plate method and the ratio method of counting as developed by Thornton and Gray (14). In the first method, Thornton's medium (13), designed to repress spreading colonies, was used. This medium contains mannitol and asparagine as sources of organic matter, and the pH was adjusted to 7.4. A few modifications were made in the ratio method of counting. It was found that the drop of liquid containing the soil and indigotin suspension dried too quickly on the slide when left exposed to the atmosphere. This resulted in a very uneven distribution of bacteria and indigotin particles on the slide and made counting under the microscope very difficult. To overcome this difficulty the slides were placed in a receptacle where the atmosphere was very humid and the films dried slowly and uniformly. Also, difficulty was experienced in staining the bacteria with the two stains used by Thornton and Gray. The bacteria were found to be stained in many cases a faint pink instead of bright red. It was found, as suggested by Fred and Waksman (4), that immersing the slides in 40% acetic acid for three minutes prior to staining remedied the difficulty to a considerable extent. Finally 32 fields were counted for each sample instead of the greater number suggested by Thornton and Gray (14).

A short comparison of the two methods of counting bacteria would perhaps not be out of place here. The ratio method was found to have certain

advantages. It proved to be much faster up to the point where the actual counts under the microscope are made. This is a decided advantage in that the slides can be prepared in a very short time and then set away to be counted later, as they keep indefinitely once the film is fixed on the slide, stained, and dried. Also, the amount of equipment needed for this method is much smaller than that required to carry out the plate method of counting. Finally, the numbers determined by the ratio method more closely approximate the total numbers of bacteria present in soil, as the counts are tremendously greater. The ratio method, however, was found to have some disadvantages. Counting was found to be tedious when compared to counting colonies on plates. The indigo particles were found to vary considerably in size and they often clumped, making it difficult to estimate the number in a clump. Also it was often difficult to distinguish between bacteria and soil particles, and when making these counts only the stained particles that could definitely be assumed to be bacteria were included.

Results

EXPERIMENT I

Nitrification and wheat yields as affected by applications of high lime Stony Plain peat and commercial fertilizers to Fallis gray wooded soil

The plan and results of this experiment are recorded in Tables I, II, and III. It should be noted that the peat was applied at the beginning of the experiment, and the commercial fertilizer at the time of seeding the first and fourth crops. Five successive crops of wheat were grown.

TABLE I

PLAN OF EXPERIMENTS ON WHEAT YIELDS AND NITRIFICATION AS AFFECTED BY APPLICATIONS OF PEAT AND COMMERCIAL FERTILIZERS TO FALLIS GRAY WOODED SOIL¹

Treatment	Composition of commercial fertilizers			Application per acre ²	
	N, %	S, %	P ₂ O ₅ , %	Fer- tilizer, lb.	Peat, lb.
Control soil					
Soil + surface peat					6000
Soil + subsurface peat					6000
Soil + ammonium phosphate	16	14	20	800	
Soil + surface peat + ammonium phosphate	16	14	20	800	6000
Soil + subsurface peat + ammonium phosphate	16	14	20	800	6000
Soil + subsurface peat + triple superphosphate	—	—	43	400	6000
Soil + subsurface peat + ammonium sulphate	20	24	—	600	6000

¹ In Experiments I, II, and IV, Stony Plain neutral (high lime) peat was used. In Experiment III Chip Lake acid peat was used.

² Rate is based on surface area of soil in pot.

Nitrates

After one month the soil treated with ammonium phosphate, with and without peat, and ammonium sulphate with peat, showed decided increases in nitrate nitrogen, whereas the soil treated with triple superphosphate and peat, and with peat alone, remained as low as the control for several months, as shown in Table II. Another significant fact brought out in Table II is that the soil treated with surface and subsurface peat is in no case much lower in nitrate than the control soil, thus indicating that the peat treatments are at least not harmful in this respect. Actually the soil treated with subsurface peat alone was distinctly higher in nitrate, on the average, than the control soil.

TABLE II

NITRATE NITROGEN IN FALLIS GRAY WOODED SOIL TREATED WITH STONY PLAIN PEAT AND VARIOUS FERTILIZERS. EXPERIMENT I

Date of sampling	Treatment							
	Control	Surface peat	Sub-surface peat	Amm. phos.	Surface peat + amm. phos.	Sub-surface peat + amm. phos.	Sub-surface peat + triple super-phos.	Sub-surface peat + amm. sul.
	Nitrate nitrogen (p.p.m.)							
Nov. /34	2.8	2.7	3.9	4.0	3.4	5.7	2.9	3.8
Dec. /34	1.5	1.4	0.8	29.6	26.7	21.6	1.1	19.5
Jan. /35	6.9	2.3	5.6	14.3	42.1	20.0	3.6	26.7
Feb. /35	19.5	11.4	19.5	42.1	38.1	25.8	16.6	38.1
Mar. /35	20.8	21.7	21.7	32.3	30.3	26.3	25.0	28.7
April /35	12.2	26.3	20.8	17.8	50.0	20.8	11.2	19.2
May /35	16.0	12.4	13.9	28.7	26.0	18.7	12.6	20.7
June /35	17.5	24.4	21.5	31.1	28.0	28.0	20.0	28.0
July /35	18.4	14.0	34.7	33.7	34.1	28.0	17.3	35.0
Aug. /35	13.2	12.0	22.4	33.3	23.3	24.9	18.3	13.7
Sept. /35	12.3	17.2	62.5	42.0	50.0	47.6	32.7	32.2
Oct. /35	23.8	18.5	37.0	50.0	50.0	43.4	25.0	28.5
Nov. /35	8.9	13.9	32.2	100.0	50.0	48.7	22.2	37.0
Dec. /35	11.1	10.6	37.0	17.9	30.3	25.3	26.3	18.8
Jan. /36	21.7	7.8	12.5	22.7	13.0	18.5	6.0	16.7
Feb. /36	33.3	30.3	25.9	100.0	62.5	32.2	31.2	39.2
Mar. /36	12.3	25.0	55.5	58.8	29.4	20.0	35.5	29.4
Nitrate nitrogen average	14.8	14.8	25.1	38.7	34.5	26.8	18.1	25.6

Moisture, %

Moisture average	15.0	17.1	16.9	15.8	18.7	16.6	17.9	19.1
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Crop Yields

In Table III, it should be noted that the first, fourth, and fifth crops (Fig. 1) were relatively good, and the second and third relatively poor. The third crop yields were reduced to some extent by insect (aphid) injury.

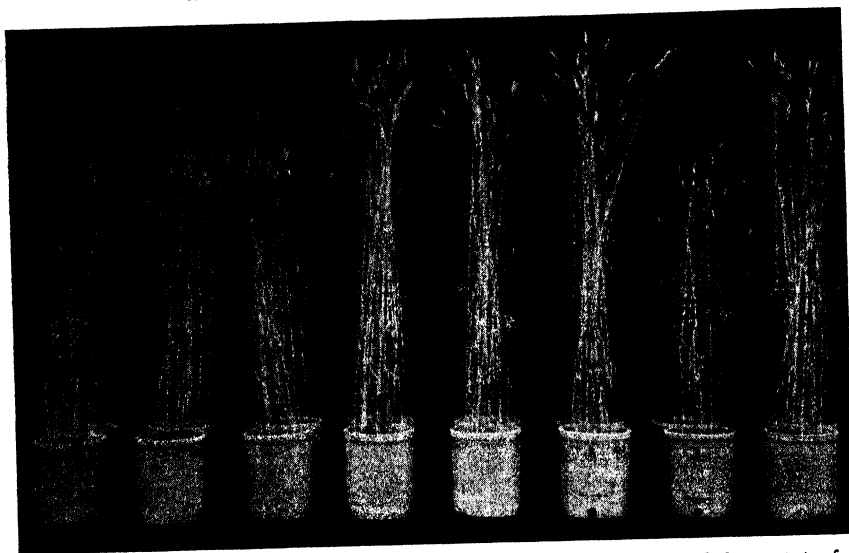


FIG. 1. Fifth crop of wheat on Fallis gray wooded soil (Experiment I). Only two pots of each treatment were photographed. Treatment: 1. Control; 2. Soil + surface Stony Plain peat; 3. Soil + subsurface Stony Plain peat; 4. Soil + ammonium phosphate; 5. Soil + surface peat + ammonium phosphate; 6. Soil + subsurface peat + ammonium phosphate; 7. Soil + subsurface peat + triple superphosphate; 8. Soil + subsurface peat + ammonium sulphate.

TABLE III

AVERAGE YIELDS OF FIRST, SECOND, THIRD, FOURTH, AND FIFTH CROPS OF WHEAT IN GRAMS PER POT, FROM FALLIS GRAY WOODED SOIL TREATED WITH STONY PLAIN PEAT AND VARIOUS FERTILIZERS. EXPERIMENT I

Treatment	1st crop ¹ Nov. /34 - April /35		2nd crop, May /35 - Aug. /35		3rd crop, Sept. /35 - Feb. /36		4th crop ¹ April /36 - Aug. /36		5th crop, Sept. /36 - Feb. /37		Average	
	Grain + straw	Grain	Grain + straw	Grain	Grain + straw	Grain	Grain + straw	Grain	Grain + straw	Grain	Grain + straw	Grain
Control soil	6.4	0.8	6.8	1.5	3.5	1.0	5.9	1.9	5.2	1.5	5.6	1.3
Soil + surface peat	7.1	1.1	5.3	0.6	4.0	1.0	7.1	2.1	5.3	1.6	5.8	1.3
Soil + subsurface peat	8.5	1.9	5.5	0.8	4.8	1.1	8.1	2.2	6.4	1.8	6.7	1.6
Soil + ammonium phosphate	17.8	2.9	7.2	0.9	4.0	0.9	13.4	3.7	12.4	3.3	11.0	2.3
Soil + surface peat + ammonium phosphate	16.0	3.2	7.7	1.1	4.0	0.9	14.5	4.6	12.9	3.8	11.0	2.7
Soil + subsurface peat + ammonium phosphate	19.0	5.2	9.9	3.4	4.4	1.2	16.9	6.0	13.6	4.0	12.8	4.0
Soil + subsurface peat + triple superphosphate	7.2	1.1	4.8	0.6	4.6	1.3	6.7	2.2	6.0	1.7	5.9	1.4
Soil + subsurface peat + ammonium sulphate	12.2	2.6	7.0	0.9	4.2	0.9	15.4	3.6	9.5	2.5	9.7	2.1
Necessary difference at 5% level of significance	2.9	1.2	2.6	1.1	0.9	0.5	1.1	0.7	0.8	0.4		

¹ Peat applied at time of seeding first crop, and commercial fertilizers at times of seeding first and fourth crops.

There were no large differences between the control yields and the yields from the surface and subsurface peat treatments, but the peat treatments gave higher average total yields in four out of five crops, and the subsurface peat gave significantly higher total yields in three out of five crops. The subsurface peat treatment in every case gave a higher average yield than the surface. The application of ammonium phosphate gave significantly higher yields than the application of peat alone in the first, fourth, and fifth crops. Ammonium phosphate gave significantly higher yields in combination with subsurface peat than alone in the first, second, fourth, and fifth crops. The pots treated with ammonium sulphate and subsurface peat gave rather smaller yields on the average than those treated with ammonium phosphate alone, and the triple superphosphate with subsurface peat treatment gave little better yields than the controls.

EXPERIMENT II

Nitrification and wheat yields as affected by applications of high lime Stony Plain peat and commercial fertilizers to Fallis gray wooded soil

Experiment II is an exact duplication of Experiment I, except that it was started at a later date. The plan and results of this experiment are recorded in Tables I, IV, and V.

TABLE IV

NITRATE NITROGEN IN FALLIS GRAY WOODDED SOIL TREATED WITH STONY PLAIN PEAT AND VARIOUS FERTILIZERS. EXPERIMENT II

Date of sampling	Treatment							
	Control	Surface peat	Sub-surface peat	Amm. phos.	Surface peat + amm. phos.	Sub-surface peat + amm. phos.	Sub-surface peat + triple super-phos.	Sub-surface peat + amm. sul.
	Nitrate nitrogen (p.p.m.)							
May /35	3.3	4.4	3.3	4.9	4.7	4.9	4.7	5.4
June /35	4.6	7.6	6.4	20.6	5.0	31.1	8.3	22.9
July /35	8.2	17.5	13.6	14.4	17.2	16.9	9.1	30.8
Aug. /35	11.7	6.8	10.2	11.2	10.0	14.2	7.9	3.3
Sept. /35	22.9	25.0	22.2	27.7	11.5	47.6	20.8	13.9
May /36	50.0	41.6	47.6	33.3	33.3	22.2	31.2	40.0
June /36	100.0	37.0	166.6	83.3	34.4	142.0	100.0	76.9
July /36	83.3	90.9	55.5	90.9	50.0	71.4	25.0	43.4
Aug. /36	55.5	47.6	71.4	50.0	52.6	54.0	20.0	55.5
Sept. /36	58.8	83.3	90.9	66.7	55.5	52.6	35.7	47.6
Nitrate nitrogen average	39.8	36.2	48.8	40.3	27.4	45.7	26.3	34.0
Moisture, %								
Moisture average	18.7	18.9	18.9	17.7	21.3	21.5	19.4	21.5

The pot cultures were maintained from May, 1935, to September, 1936, but nitrates were determined only between May and September each year. Peat and fertilizers were added at the beginning of the experiment only and four crops of wheat were grown in succession.

Nitrates

After one month, as in Experiment I, the gray soil treated with ammonium phosphate and sulphate contained much more nitrate than the untreated, with one exception (Table IV). The exception was the soil treated with surface peat and ammonium phosphate, and in this experiment there is some indication that surface peat tends to depress nitrate accumulation. After two months the nitrate content was not so definitely related to the original applications of ammonium fertilizers. The nitrate content of the soil treated with subsurface peat, on the other hand, was very high in the latter half of this experiment, even greater on the average than that of soil treated with both ammonium phosphate and subsurface peat. The triple superphosphate and subsurface peat treatment gave the lowest average nitrate content in the series, and for this effect no satisfactory explanation is offered.

Crop Yields

The wheat yields of Experiment II are shown in Table V. Significant differences were obtained in the first and third crops, but not in the second and fourth. In the first crop the ammonium phosphate alone, the ammonium

TABLE V

AVERAGE YIELDS OF FIRST, SECOND, THIRD, AND FOURTH CROPS OF WHEAT IN GRAMS PER POT, FROM FALLIS GRAY WOODED SOIL TREATED WITH STONY PLAIN PEAT AND VARIOUS FERTILIZERS.

EXPERIMENT II

Treatment	1st crop, May /35 - Aug. /35		2nd crop, Oct. /35 - Feb. /36		3rd crop, April /36 - Aug. /36		4th crop, Aug. /36 - Feb. /37		Average	
	Grain + straw	Grain	Grain + straw	Grain	Grain + straw	Grain	Grain + straw	Grain	Grain + straw	Grain
Control soil	9.2	3.4	3.7	1.1	7.0	2.6	7.7	2.2	6.9	2.3
Soil + surface peat	9.0	3.7	3.5	0.9	7.7	3.0	8.4	2.6	7.1	2.5
Soil + subsurface peat	7.9	3.4	4.0	1.1	8.5	3.3	7.1	2.1	6.9	2.5
Soil + ammonium phosphate	11.8	4.8	3.6	1.0	8.6	3.4	7.1	2.1	7.8	2.8
Soil + surface peat + ammonium phosphate	9.8	5.0	3.7	1.0	8.7	2.9	7.2	1.9	7.3	2.7
Soil + subsurface peat + ammonium phosphate	12.0	4.8	4.3	1.1	11.6	4.5	7.3	2.2	8.8	3.1
Soil + subsurface peat + triple superphosphate	9.2	3.7	3.4	0.8	10.3	4.1	7.1	2.0	7.5	2.6
Soil + subsurface peat + ammonium sulphate	12.3	4.9	3.5	0.8	8.0	2.9	7.6	2.2	7.8	2.7
Necessary difference at 5% level of significance	3.0	1.0	0.9	0.3	1.0	0.8	1.9	0.7		

¹ Peat and commercial fertilizers applied at time of seeding first crop.

phosphate with surface and subsurface peat, and the ammonium sulphate with subsurface peat, gave significant increases in yield over the control. In the third crop all of the treatments except surface peat alone gave significant increases in yield. As in Experiment I, the highest yields of the four successive crops on the average were given by the ammonium phosphate with subsurface peat.

EXPERIMENT III

Nitrification and wheat yields as affected by applications of acid Chip Lake peat and commercial fertilizers to Fallis gray wooded soil

Experiment III differs from the two previous experiments in one respect: peat with a distinctly acid reaction was used instead of high lime peat. The plan and results of this experiment are recorded in Tables I, VI, and VII.

TABLE VI

NITRATE NITROGEN IN FALLIS GRAY WOODED SOIL TREATED WITH CHIP LAKE PEAT AND VARIOUS FERTILIZERS. EXPERIMENT III

Date of sampling	Treatment							
	Control	Surface peat	Sub-surface peat	Amm. phos.	Surface peat + amm. phos.	Sub-surface peat + amm. phos.	Sub-surface peat + triple super-phos.	Sub-surface peat + amm. sul.
	Nitrate nitrogen (p.p.m.)							
June /35	10.8	8.4	9.0	8.4	7.6	7.7	6.7	7.6
July /35	7.0	7.2	11.2	11.9	17.7	11.0	5.8	40.1
Aug. /35	8.3	6.4	6.8	30.3	35.5	32.3	8.1	66.6
Sept. /35	7.8	6.9	19.2	38.1	31.2	28.5	10.2	93.0
Oct. /35	17.5	11.6	13.8	43.4	43.4	41.6	22.2	96.1
Nov. /35	7.8	22.7	12.3	31.2	22.2	80.0	16.7	8.2
Dec. /35	25.0	10.0	15.6	22.7	16.1	15.6	15.2	66.6
Jan. /36	19.2	15.2	13.3	35.7	22.2	31.3	18.9	138.8
Feb. /36	7.1	8.3	7.1	6.5	16.6	10.5	11.9	16.6
Mar. /36	3.9	4.5	3.8	6.3	21.7	3.2	20.0	20.8
April /36*	41.6	45.4	22.7	40.0	47.6	30.3	27.0	55.5
May /36	40.0	41.6	66.6	60.6	58.8	33.3	34.5	71.4
June /36	33.3	17.9	52.6	50.0	111.1	83.3	25.0	100.0
July /36	66.6	76.9	43.4	125.0	—	76.9	32.2	47.6
Aug. /36	56.6	76.9	52.6	125.0	111.1	125.0	50.0	125.0
Sept. /36	20.0	55.5	51.3	90.9	62.5	70.4	37.0	90.9
Nitrate nitrogen average	23.3	26.0	25.1	45.4	41.7	42.6	21.3	58.3
Moisture, %								
Moisture average	13.7	16.5	15.6	15.3	16.4	16.3	15.6	16.0

* A second application of commercial fertilizer only was made at this time.

Pot cultures were maintained from June, 1935, to February, 1937. Four successive crops of wheat were grown during this period, but the monthly nitrate determinations were stopped in September, 1936. Peat and fertilizers were added at the beginning of the experiment and a second application of fertilizers only in April, 1936, at the time of seeding the third crop.

Nitrates

In Experiment III (as in Experiment I) there is no evidence that applications of surface or subsurface peat depress nitrification (Table VI). Ammonium phosphate and surface peat produced about the same increase in average nitrate content as ammonium phosphate and subsurface peat but the increase was less than that produced by ammonium phosphate alone. The highest average nitrate was given by ammonium sulphate with subsurface peat. Triple superphosphate and subsurface peat again gave a low average nitrate content, ranking just below that of the control.

Crop Yields

The wheat yields of Experiment III are shown in Table VII. Significant differences were obtained in all four crops, but the yields of the second crop were poor. The crop yields, when averaged, show the same general trends as in Experiments I and II.

TABLE VII

AVERAGE YIELDS OF FIRST, SECOND, THIRD, AND FOURTH CROPS OF WHEAT IN GRAMS PER POT, FROM FALLIS GRAY WOODED SOIL TREATED WITH CHIP LAKE ACID PEAT AND VARIOUS FERTILIZERS. EXPERIMENT III

Treatment	1st crop ¹ , June /35 – Sept. /35		2nd crop, Oct. /35 – Feb. /36		3rd crop ¹ , April /36 – Aug. /36		4th crop, Aug. /36 – Feo. /37		Average	
	Grain + straw	Grain	Grain + straw	Grain	Grain + straw	Grain	Grain + straw	Grain	Grain + straw	Grain
Control soil	6.6	2.3	2.2	0.3	8.9	2.9	5.5	1.8	5.8	1.8
Soil + surface peat	8.8	3.2	2.1	0.4	6.4	1.9	7.7	2.5	6.2	2.0
Soil + subsurface peat	11.3	6.7	2.1	0.5	6.6	2.2	6.6	2.2	6.6	2.9
Soil + ammonium phosphate	12.3	6.8	2.9	0.7	12.3	5.3	11.1	3.6	9.6	4.1
Soil + surface peat + ammonium phosphate	16.6	6.8	2.4	0.6	12.0	5.1	11.9	3.8	10.7	4.1
Soil + subsurface peat + ammonium phosphate	18.2	7.8	2.5	0.7	13.1	6.0	10.6	3.3	11.1	4.4
Soil + subsurface peat + triple superphosphate	12.5	4.5	2.7	0.7	6.6	2.5	5.2	1.5	6.7	2.3
Soil + subsurface peat + ammonium sulphate	18.2	6.4	2.8	0.7	8.6	3.4	11.4	3.5	10.2	3.5
Necessary difference at 5% level of significance	4.5	1.3	0.4	0.2	1.2	0.9	1.9	0.7		

¹ Peat applied at time of seeding first crop, and commercial fertilizers at times of seeding first and third crops.

The ammonium phosphate alone and in combination with surface and subsurface peat gave significant increases in yield in all four crops, and the ammonium sulphate in combination with subsurface peat gave significant increases in three out of four crops. Treatment with triple superphosphate combined with subsurface peat gave about the same average increase as treatment with subsurface peat alone. Peat alone increased the yields somewhat and, as in Experiment I, subsurface peat gave larger average increases than surface. The ammonium phosphate in combination with subsurface peat gave a significantly higher yield than ammonium phosphate alone in the first crop, and, as in Experiments I and II, the highest average total and grain yields for all crops were given by this combination.

EXPERIMENT IV

Effects of applications of high lime Stony Plain peat and commercial fertilizers on bacterial numbers in Fallis gray wooded soil

This experiment was carried out with the object of finding out how treatments of peat and commercial fertilizers would affect the numbers of bacteria in gray wooded soil. As stated in the outline of the investigation, the bacterial numbers were determined by the plate method first; then the experiment was repeated and the numbers were determined by the ratio method.

The results of the counts made by the plate method are shown in Table VIII. The numbers did not increase much at any time in the control soil. In the treated soil the highest counts were obtained after two, three, or four months' incubation, after which time the numbers decreased. The highest average count for the entire period of incubation was given by ammonium phosphate combined with subsurface peat, the second highest by ammonium phosphate with surface peat, and the third highest by ammonium sulphate with subsurface peat. The differences between the average counts given by treatment with ammonium phosphate, surface peat, subsurface peat, and triple superphosphate combined with subsurface peat, were small.

In studying Table IX the first and most striking feature is the great number of bacteria shown by the ratio method. Instead of the bacterial numbers ranging about 500,000 to about 8,000,000 per gm. of soil as shown by the plate count method, the numbers varied as a rule from about 200,000,000 to 2,000,000,000 by the ratio method. Thus it is evident that only a very small fraction of the bacteria actually present in the soil will grow on ordinary nutrient media.

Table IX shows that the bacterial numbers were considerably higher in the soil treated with peat and commercial fertilizers than in the control soil. The soil treated with subsurface peat and ammonium phosphate was the first to show high numbers of bacteria and contained the highest average number of bacteria. The averages show that the surface peat plus ammonium phosphate ranks second and the subsurface peat plus ammonium sulphate third in bacterial numbers. The differences in average counts between the

TABLE VIII

NUMBERS OF BACTERIA DETERMINED BY PLATE COUNT METHOD IN FALLIS GRAY WOODED SOIL TREATED WITH STONY PLAIN PEAT AND VARIOUS FERTILIZERS. EXPERIMENT IV

Date of sampling, 1935	Treatment							
	Control	Surface peat	Sub-surface peat	Amm. phos.	Surface peat + amm. phos.	Sub-surface peat + amm. phos.	Sub-surface peat + triple super-phos.	Sub-surface peat + amm. sul.
	Bacteria (millions per gram)							
May 28	2.1	1.7	1.4	2.2	1.7	1.6	1.7	1.3
June 11	1.8	2.5	2.8	2.2	2.7	3.6	2.3	2.6
June 25	1.7	4.6	4.6	3.3	4.1	7.0	3.3	3.0
July 9	1.6	2.6	1.9	3.2	4.3	4.2	2.7	3.4
Aug. 6	2.2	3.7	4.3	4.3	7.6	5.9	5.1	7.3
Aug. 20	1.0	1.1	1.6	1.3	1.5	1.4	0.8	1.5
Sept. 3	1.1	1.6	0.9	1.3	2.7	1.8	1.1	1.7
Sept. 17	1.5	2.3	2.6	2.6	1.9	2.6	1.8	2.8
Oct. 1	0.6	0.8	0.7	1.3	1.4	1.9	0.9	1.2
Oct. 15	1.7	1.9	2.1	2.2	1.9	1.9	1.8	2.0
Average number	1.5	2.3	2.3	2.4	3.0	3.2	2.1	2.7

TABLE IX

NUMBERS OF BACTERIA, DETERMINED BY DIRECT RATIO METHOD OF COUNTING, IN FALLIS GRAY WOODED SOIL TREATED WITH STONY PLAIN PEAT AND VARIOUS FERTILIZERS. EXPERIMENT IV

Date of sampling, 1936	Treatment							
	Control	Surface peat	Sub-surface peat	Amm. phos.	Surface peat + amm. phos.	Sub-surface peat + amm. phos.	Sub-surface peat + triple super-phos.	Sub-surface peat + amm. sul.
	Bacteria (millions per gram)							
June 24	373	421	545	601	370	343	430	241
July 8	476	971	760	492	627	446	342	499
July 22	315	465	603	763	653	748	462	460
Aug. 6	294	438	575	442	672	1216	412	787
Aug. 20	198	428	433	476	758	756	578	762
Sept. 3	850	1013	1048	1966	1982	2016	1260	1613
Oct. 12	41	329	694	578	1219	1181	1085	1087
Oct. 26	452	610	867	595	826	870	522	803
Nov. 2	223	244	280	238	304	290	289	405
Nov. 16	178	272	317	277	250	254	390	249
Average number	340	519	612	643	766	812	577	691

other treated plots are not significant. The bacterial counts were found to reach a maximum about three months after the application of the peat and fertilizers. From then on the bacterial numbers began to level out until by the end of the experiment, which was carried on for about five months, there were no large differences between the different treatments.

With one unimportant exception the averages given in Tables VIII and IX show that the bacterial numbers as affected by treatment rank in the same order in the ratio as in the plate count method. The same general conclusions regarding effects of amendments will therefore hold.

EXPERIMENT V

Effect of peat, straw, and cotton on nitrate nitrogen content of mineral soils

Because a great deal of experimental work has shown that certain kinds of organic matter when added to mineral soil depress the nitrate nitrogen content for a time, and because in this investigation peat was found to have rather the opposite effect, it was felt necessary to set up an experiment where surface Stony Plain peat high in cellulose could be compared with straw and cotton, also high in cellulose. These forms of organic matter were added separately and in combination with ammonium phosphate. Two different types of soil were used, a black fertile soil and a gray wooded relatively infertile soil. The results of the experiment are shown in Tables X and XI.

When mixed with gray wooded soil, surface peat, although high in cellulose did not cause any significant decrease in nitrate accumulation at first although some increase was produced later. Straw caused a decided decrease at first, but a recovery was made after the first month and gains counterbalanced the earlier losses. Cotton reduced the nitrate to practically nothing for a period of about three months. Ammonium phosphate greatly increased nitrate accumulation, and when ammonium phosphate was added together with organic matter, nitrate accumulation was greatly retarded by cotton, but not by straw or peat.

In the case of the black soil the trends are somewhat the same, although the applications of straw and cotton do not appear to be quite so harmful as in the case of the gray soil. The peat did not cause any significant decrease in nitrate accumulation, whereas the straw caused a small decrease at first and the cotton caused considerable decrease (Table XI). Nitrate accumulation was greatly stimulated by the addition of ammonium phosphate alone, and when ammonium phosphate was added together with organic matter, nitrate accumulation apparently was not retarded by the peat, straw, or cotton.

Discussion

A great deal of experimental work on the reclamation of peat has been done in Europe and America, but comparatively few investigations on the direct application of peat to improve mineral soils have been reported. It is interesting to note that the opposite practice, that of applying mineral soil or sand to peat, in order to improve the peat, is well established in Europe.

TABLE X

NITRATE NITROGEN IN FALLIS GRAY WOODED SOIL TREATED WITH STONY PLAIN PEAT, WHEAT STRAW, COTTON, AND AMMONIUM PHOSPHATE FERTILIZER. EXPERIMENT V

Date of sampling	Treatment							
	Control	Surface peat	Amm. phos.	Surface peat + amm. phos.	Straw	Straw + amm. phos.	Cotton	Cotton + amm. phos.
	Nitrate nitrogen (p.p.m.)							
Dec. 4/35	10.0	8.6	8.6	9.1	6.3	4.2	6.0	3.5
Dec. 13/35	8.1	11.9	10.0	21.3	Trace	13.2	Trace	6.8
Jan. /36	6.1	6.9	33.3	47.6	5.1	47.6	Trace	21.7
Feb. /36	15.2	11.4	44.4	43.4	13.9	45.4	0.0	6.7
Mar. /36	9.2	31.4	40.0	47.6	12.8	12.5	7.6	32.7
April /36	33.3	40.0	108.6	89.2	23.8	58.8	12.5	50.0
May /36	25.0	35.7	23.8	54.0	38.5	20.0	12.8	25.0
June /36	39.2	29.4	105.0	31.2	90.9	71.4	45.4	31.2
July /36	166.7	47.6	192.3	312.5	138.9	250.0	78.1	100.0
Aug. /36	90.9	66.6	83.3	111.1	71.4	125.0	35.7	71.4
Sept. /36	71.4	156.3	178.6	208.3	83.3	104.2	62.5	104.2
Nitrate nitrogen average	43.2	40.5	75.3	88.7	44.1	68.4	23.7	41.2

Moisture, %

Moisture Average	14.9	15.2	16.0	16.3	16.7	17.3	17.3	18.7
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TABLE XI

NITRATE NITROGEN IN EDMONTON BLACK SOIL TREATED WITH STONY PLAIN PEAT, WHEAT STRAW, COTTON, AND AMMONIUM PHOSPHATE FERTILIZER. EXPERIMENT V

Date of sampling	Treatment							
	Control	Surface peat	Amm. phos.	Surface peat + amm. phos.	Straw	Straw + amm. phos.	Cotton	Cotton + amm. phos.
	Nitrate nitrogen (p.p.m.)							
Dec. /35	33.3	50.0	15.6	14.0	22.7	33.3	21.7	23.8
Jan. /36	48.7	51.2	104.0	55.5	40.8	89.2	23.8	89.2
Feb. /36	46.8	37.5	75.0	96.1	30.8	166.6	37.5	62.5
Mar. /36	27.7	22.7	50.0	76.9	45.4	50.0	31.3	119.0
April /36	34.4	27.7	76.9	131.5	35.7	62.5	27.7	40.0
May /36	45.4	55.5	55.5	66.6	66.6	71.4	34.4	62.5
June /36	131.5	108.6	156.2	156.2	125.0	138.8	86.2	131.5
Nitrate nitrogen average	52.5	50.4	76.2	85.3	52.4	87.4	37.5	75.5
	Moisture, %							
Moisture average	32.8	33.6	35.0	36.5	33.8	31.6	30.8	33.4

The investigation reported in this paper was concerned chiefly with chemical, microbiological, and general fertility questions. No attempt was made to cover the physical problems. However, it is felt that if the physical side were studied, the application of peat to gray wooded soils would be found to be decidedly beneficial from this standpoint, because it was noticed in handling the soil that where peat was applied there appeared to be more structure, less tendency to bake, and a greater moisture holding capacity. Dachnowski-Stokes (3) states that the application of poorly decomposed peat to soils, where there is a danger of drought, and where rainfall is light during the summer months, will do much to improve their structure and moisture holding capacity.

The fertilizer treatment plan of the experiment was not made more complete because of the labour involved in carrying out a more complete plan. The one fertilizer that was applied alone, as well as in combination with neutral and acid surface and subsurface peat, was ammonium phosphate 16-20. This fertilizer contains sulphur as well as nitrogen and phosphorus, and is one of the best general purpose fertilizers for gray wooded soils.

These experiments show that from a chemical standpoint the application of such peats as were used is definitely not harmful, but rather beneficial. This is noteworthy because some experimenters have found that other forms of organic matter, with as high a carbon:nitrogen ratio as that of some of the peat used, tend to reduce the nitrate content of the soil. Previous investigations (10) showed that nitrate accumulation occurred in Alberta peat in spite of the peat's low nitrogen content, or high carbon:nitrogen ratio. This may be due to differences in availability of carbon and nitrogen, for, as pointed out by Norman (11), if a portion of the carbon is relatively unavailable the true ratio may be considerably narrower. McCool (7) found that nitrate formation took place in peat and that peat was not inert in this respect. Baur (2) reported that in composting a very acid undecomposed peat nitrate accumulation took place if lime were added, and also that accumulation took place more quickly where a nitrogen-phosphorus-potassium fertilizer was added together with lime.

Scott (12) and other workers state that the application of wheat straw to soil causes a marked decrease in nitrates for a certain period of time. In the experiments reported in this paper it was shown that straw causes some decrease in nitrate when first applied to gray soil, and cotton, a relatively pure form of cellulose, causes a great decrease. Anderson (1) states that nitrification can proceed in the presence of cellulose, but unless nitrogen is present in quantities above that required by the bacteria, the nitrates are utilized as rapidly as they are formed. Other investigators (working with such materials as wheat straw) have found that nitrogen of organic matter in soils can appear as nitrate only if the carbon:nitrogen ratio is 12 or less.

McCool (7) reports that a combination of peat and fertilizer salts proved beneficial to unproductive surface sandy loam soil. Furthermore he states that a satisfactory soil may be made up by using a good grade of peat, commercial fertilizers, and a poor surface soil, sand, or subsoil. The experiments reported in this paper show that wheat crop yields were generally increased somewhat by peat alone (especially by subsurface peat), and increased substantially by ammonium phosphate alone, but that the largest increases in all three series were produced by a combination of ammonium phosphate and subsurface peat.

In previously reported experiments with Alberta peats (8, 10) it was shown that large increases in bacterial numbers (determined by plate counting) were produced by the addition of certain mineral fertilizers to peat soil. Baur (2) found that in composting acid peat the bacterial numbers were increased substantially, and that in completely fertilized and limed samples the numbers were increased still more. The experiments reported in this paper show that the peat alone and the ammonium phosphate alone produced some increase in bacterial numbers, but that the largest increases as determined by both the plate count and ratio methods, were given by treatment with a combination of peat and ammonium phosphate. The numbers of bacteria shown by the ratio method were very great. Instead of ranging from about 500,000 to about 8,000,000 per gm. of soil as in the plate count method, the numbers varied from about 200,000,000 to 2,000,000,000 in the ratio method.

Some field tests of peat and commercial fertilizers, applied separately and in combination, as amendments for gray wooded soil, have been carried on for several seasons, and will be published at a later date.

Acknowledgments

The writers wish to express their appreciation to Dr. F. A. Wyatt, Head of the Department of Soils, for counsel and support of the work, to Dr. J. W. Hopkins of the National Research Council, for advice regarding statistical treatment of yield data, and to the Carnegie Research Grants Committee of the University of Alberta for necessary financial assistance.

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CAROTENE IN FEED GRASSES¹

BY JOHN ALLARDYCE² AND DOUGLAS MILSOM³

Abstract

Cuttings of feed grasses less than 7 in. high, taken from the same field and given the same treatment, were found to vary considerably in carotene content. Factors that were found to contribute to these variations were: the amount of sunshine and rain prior to each cutting, the height of the cutting, and the manner of storage. Higher carotene content was found when the cuttings were taken following periods of increased daily sunshine particularly if these periods were preceded by rain. Cuttings less than 7 in. high contained larger amounts of carotene than did 12 in. cuttings. Storing the ground, dehydrated feed grasses in 100-lb. paper-lined burlap sacks at 35° F. during the summer months reduced the average loss in carotene content to 7.5%.

Introduction

The value of alfalfa in live-stock feeds has long been recognized. Today on the Pacific coast of Canada and of the United States the price paid by feed companies for alfalfa and feed grasses in general is based primarily on carotene content. Findings reported by Crabtree (2) indicate that field grasses, grass and clover mixtures, and oat grass are comparable with alfalfa in carotene content. In 1938, Johnson (6) reported the carotene value for green alfalfa as 22.7 mg. per 100 gm. dry matter. This is in keeping with the 15 to 25 mg. values found by Milsom (7) in 1940. More recently Crabtree (2) has confirmed Milsom's findings, and reports greater yields of carotene (35 to 39 mg. per 100 gm.) in both alfalfa and grass crops on which fertilizers were used.

In British Columbia, field grasses are easily obtained and require little cultivation especially if grown on delta land. In the Sumas area they have to some extent begun to replace alfalfa as a source of carotene (2). It has been noted that cuttings taken at different times from the same field may show considerable variation in carotene content and consequently will bring different prices to the farmer.

In the present investigation, these variations have been studied in relation to the natural conditions that occur during production and storage of these feeds. Variables considered as possibly affecting the carotene content include hours of sunshine, temperature range, rainfall, height of grass at cutting, and storage conditions.

Von Hausen (5) in 1933 investigated the effect of varying the nitrogen-potassium-phosphorus ratio on the carotene content and came to the conclusion that both the amount and ratio of these elements were important factors. The ratio producing maximum growth of greenstuff also produced the highest carotene content.

¹ Manuscript received May 23, 1941, and as revised September 29, 1941.
Contribution from the Department of Biology and Botany, University of British Columbia, Vancouver, B.C., with financial assistance from Buckenfield's Ltd., Vancouver.

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Fraps and Kemmerer (4) investigated carotene losses in alfalfa and feed mixtures during storage and found these much higher in summer than in winter. In alfalfa placed in open jars at room temperature for eight weeks, carotene losses ranged from 6 to 70%. Under like conditions, at 33° to 35° F., the losses ranged from 0 to 26%.

Fagan and Ashton (3) in 1938 studied various methods of drying grasses and their effects on the carotene content. They found that natural field drying causes losses as high as 66%. This loss was cut approximately in half by the use of mechanical driers. The process consisted in passing the freshly cut grass through a chamber held at temperatures ranging from 1000° to 1600° F. and at speeds that required from two to five minutes. Minimum losses occurred when the drier was held at 1600° F. and the grass run through in two minutes.

Procedure

The grasses used in the investigation were grown on the Vancouver City Airport, delta land on Sea Island at the mouth of the Fraser River. The field grasses are those common to this area and include Yorkshire fog (*Holcus lanatus*), Kentucky blue (*Poa pratensis*), bent (*Agrostis*), and timothy (*Phleum pratense*), together with a small amount of white clover (*Trifolium repens*) and red clover (*Trifolium pratense*).

Cuttings of grass less than 7 in. high were made from June 12 to September 12 from the whole field, except a portion permitted to grow to a height of 12 in. The carotene content of this grass was compared with that of a 7-in. cutting made on the same day from the same field.

All cuttings were dehydrated by heating in a drier at 1400° F. for one minute and were then freely ground in a process requiring two minutes. The grass was then taken to the mill where it was reground and the whole cutting was thoroughly mixed before sacking in 100-lb. paper-lined burlap sacks. Samples drawn during the sacking process were thoroughly mixed and samples were removed for the determination of carotene by the method of Bolin and Khalapur (1). The 100-lb. sacks were stored at 35° F. during the summer months. On July 6, samples for carotene determination were withdrawn from sacks of the ground, dried grasses taken at random. Four subsequent determinations were made on each of the sacks.

Records of weather conditions over the experimental period were obtained from the Dominion Meteorological Station situated in the area under investigation.

The carotene value for each cutting made during the experimental period was plotted against the day of cutting and compared with the average number of hours of sunshine on the day of cutting, on one, two, three, four, and five days previous to cutting, and for the whole interval between consecutive cuttings. The sunshine curve finally used (Fig. 1) was based on the average daily sunshine for the four days previous to cutting and, since the intervals between cuttings were not always the same, the average was determined not

only for the day of cutting but for each day of the experimental period. Hence each point on the sunshine curve represents the average hours of sunshine per day for the previous four days.

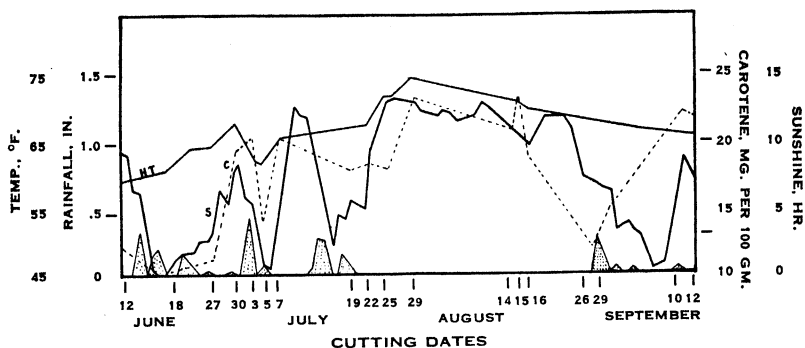


FIG. 1. C = carotene; S = sunshine; HT = maximum temperature; [stippled area] = rainfall.

Results and Discussion

In Table I and Fig. 1 appear the carotene values of cuttings made on the dates shown. Analyses were made within three days of each cutting on the ground, dried samples.

TABLE I
CAROTENE LEVELS DURING EXPERIMENTAL PERIOD

Date	Carotene, mg. per 100 gm.	Date	Carotene, mg. per 100 gm.	Date	Carotene, mg. per 100 gm.
June:		July:		August:	
12	12.2	7	20.8	15	23.9
18	10.0	19	18.2	16	19.1
27	11.2	22	18.7	26	12.0
30	19.8	25	18.3	29	15.2
		29	23.9		
July:		August:		September:	
3	20.8			10	22.7
5	14.3	14	21.4	12	22.2

As may be seen from Fig. 1, both the carotene and the sunshine curves begin and end with a peak. Each shows a second peak at the end of June, a third in the first part of July, and a more or less sustained peak from the middle of July to the middle of August. While these trends are not close enough to allow a mathematical correlation, they would seem to indicate that when the average daily sunshine for the four days previous to cutting was high, the carotene content was high.

Other factors, however, are evidently at work. From July 20 to August 26, there was a hot dry period. About the middle of August the carotene curve

began to drop—a week before the sunshine curve. At the bottom of the carotene drop it began to rain. Despite the continued decrease in sunshine over the next 10 days, and rain for a week, the carotene content rose to a new peak with the next sunshine peak. It will thus be noted that rain followed by sunshine, which stimulates plant growth, also stimulated increased production of carotene. Moreover, peaks in the maximum temperature curve appear to coincide with or slightly precede the peaks in the carotene curve.

It is to be noted that since the whole experimental area of any given height was cut on the same day and the cuttings were always given the same treatment in drying and grinding and then were thoroughly mixed before samples were drawn, variations in the mineral distribution over the area or in the distribution of the grasses and the clover found growing there cannot account for the change in the carotene content at different times of cutting.

Carotene determinations made on feed grasses cut at heights of 7 and 12 in. on the same day from the same field were 22.9 and 11.3 mg. per 100 gm., respectively.

TABLE II

CAROTENE CONTENT OF DRIED GRASS AFTER STORAGE AT 35° F., MG. PER 100 GM. DRIED GRASS

Sack	July 6	July 19	Aug. 2	Aug. 23	Sept. 6
1	13.97	13.68	13.54	13.54	13.09
2	16.03	15.89	15.89	15.72	15.15
3	10.00	9.86	9.86	9.86	8.82
4	12.06		11.92	11.92	11.77
5	9.12	9.12	9.12	9.12	9.12
6	8.09	7.94	8.09	8.09	8.09
7	10.30	10.00	9.77	9.50	8.83
8	23.98	22.60	21.62	21.62	19.42

The average loss of carotene from all sacks of stored feed grasses tested was 7.5%. The results for the first eight of these sacks appear in Table II. From a comparison of the average value with the results obtained by Fraps and Kemmerer (4) and Fagan and Ashton (3), it would appear that excessive losses in carotene content of feed grasses during the summer months can be prevented by the grinding and drying process. More recently Crabtree (2) has found that during storage of alfalfa and feed grasses under similar conditions, greater losses of carotene occurred in the alfalfa.

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ZYGOSACCHAROMYCES NECTAROPHILUS N. SP. AND ZYGOSACCHAROMYCES RUGOSUS N. SP.¹

By A. G. LOCHHEAD²

Abstract

Two species of osmophilic yeasts, previously described but not named, have been designated respectively *Zygosaccharomyces nectarophilus* Lochhead and Farrell and *Zygosaccharomyces rugosus* Lochhead and Farrell.

Introduction

During the course of previous studies in this laboratory on osmophilic yeasts causing fermentation in solutions of high sugar concentration, various species of *Torulopsis*, *Saccharomyces*, *Schizosaccharomyces*, and *Zygosaccharomyces* were isolated and described. The yeasts studied included previously described species, as well as several new species, two of which were named *Zygosaccharomyces nussbaumeri* Lochhead and Heron and *Zygosaccharomyces richteri* Lochhead and Heron, respectively (3).

Two other species of *Zygosaccharomyces*, isolated from a variety of sources during the investigations, and identified only by number, were believed to be new species not elsewhere described, a view that later studies have confirmed. In the manuscript of an "Annual Report", prepared some years later, these yeasts were accordingly given the names *Zygosaccharomyces nectarophilus* Lochhead and Farrell and *Zygosaccharomyces rugosus* Lochhead and Farrell, and type cultures were sent to the Centraalbureau voor Schimmelcultures at Baarn. In view of the fact that the manuscript referred to was not published, and the names, therefore, have not yet effectively reached the literature, the new species are hereby designated.

Zygosaccharomyces nectarophilus has been described (with illustrations) as cultures N4 (3), S3B2 (1), and 155Y (2), and *Zygosaccharomyces rugosus* as cultures S3B11 (1) and 139 (2). To the earlier morphological and physiological descriptions of these yeasts, it is now possible to add further characterization based on recent comparative studies of the nutritive requirements of osmophilic yeasts (4). For growth of *Z. rugosus*, biotin is the essential growth factor, though development is stimulated by pantothenic acid. Inositol, thiamin, and pyridoxin are non-essential nutritives. In the case of *Z. nectarophilus* both biotin and pantothenic acid are necessary for multiplication, with inositol an important additional factor in providing optimum growth.

¹ Manuscript received December 8, 1941.

Contribution No. 140 (Journal Series) from the Division of Bacteriology and Dairy Research, Science Service, Department of Agriculture, Ottawa, Canada.

² Dominion Agricultural Bacteriologist.

Description of Species

Zygosaccharomyces nectarophilus n. sp. Lochhead and Farrell

In young cultures cells round to slightly oval, generally 3 to 5 μ in diameter, with extremes of 2 to 6 μ . In old cultures "giant" cells may be seen, 9 to 10 μ in diameter. Reproduces asexually by budding, and by isogamic copulation resulting in ascospore formation. The ascus contains one to four spores, usually two to three. Spores round to slightly oval, mostly 3.5 to 4 μ in diameter, with variations between 3 and 4.5 μ . The yeast is able to develop in solutions of high sugar concentration. Produces alcoholic fermentation in dextrose, levulose, and to a lesser degree in mannose. Does not ferment arabinose, xylose, galactose, saccharose, maltose, lactose, raffinose, dextrin, mannitol, dulcitol, or salicin. Giant colony raised with little spreading, irregular in outline with broken surface. Growth scanty on carrot and potato. Induces no change in milk, and does not liquefy gelatine. Has been isolated from floral nectar, honey, and soil. Requires biotin, pantothenic acid, and inositol for normal growth.

Zygosaccharomyces rugosus n. sp. Lochhead and Farrell

In young cultures cells oval to round, the majority 4 to 6 μ in length and 3 to 4 μ wide. Reproduces asexually by budding and by isogamic copulation resulting in ascospore formation. The ascus contains one to four spores, usually two to three. Spores slightly oval to round, the majority 3 to 3.5 μ in length. The yeast is able to develop in solutions of high sugar concentration. Causes alcoholic fermentation of dextrose, levulose, mannose, saccharose, and maltose. Does not ferment arabinose, xylose, galactose, lactose, raffinose, dextrin, mannitol, dulcitol, or salicin. Giant colony round, spreading, with surface becoming covered with coarse folds. Growth abundant on carrot and potato. Causes no change in milk, but liquefies gelatine slowly. Has been isolated from honey and soil. Requires biotin and pantothenic acid for optimum growth; inositol not essential.

Descriptions of the two species in Latin are given herewith.

Zygosaccharomyces nectarophilus sp. nov.

In culturis juvenilibus, cellulis rotundatis vel leviter ovalibus, 3-5 μ (2-6 μ); in culturis senioribus per occasionem cellulis giganteis, 9-10 μ . Regenerat et gemmis et copulatione aequarum cellularum ascosporis deinde orientibus. Ascis 1-4 (plerumque 2-3)-sporis; sporis rotundatis vel leviter ovalibus, 3.5-4 μ (3-4.5 μ). In diluto forti sacchari crescere potest. Inducit fermentationem alcoholicam in dextrosio, levulosio, et in minore modum in mannoso sed nullam in arabinosio, xylosio, galactosio, saccharosio, maltosio, lactosio, raffinosis, dextrino, mannitolo, dulcitol, vel salicino. Gigantea colonia elevata, compacta, superficie fracta, ambitu irregulari; incremento parvo in segmentis radicis *Dauci Carolae* et tuberum *Solani tuberosi*. Vicissitudinem in lacte nec inducit nec gelatinam liquefacit. Segregatus e nectari florum, melle et solo. Pro incremento normali postulat biotinum, acidum pantothenicum, et inositolum.

Zygosaccharomyces rugosus, sp. nov.

In culturis juvenilibus, cellulis ovalibus vel rotundatis, fere 4-6 μ longis, 3-4 μ latis. Regenerat et gemmis et copulatione aequarum cellularum ascosporis deinde orientibus. Ascis 1-4 (plerumque 2-3)-sporis; sporis leviter ovalibus vel rotundatis, fere 3-3.5 μ longis. In diluto forti sacchari crescere potest. Inducit fermentationem alcoholicam in dextrosio, levulosio, mannoso, saccharosio, maltosio sed nullam in arabinosio, xylosio, galactosio, lactosio,

raffinosis, dextrino, mannitolo, dulcitol, vel salicino. Gigantea colonia rotunda, expansa superficie crasse plicata; incremento amplo in segmentis radicis *Dauci Carolae* et tuberum *Solani tuberosi*. Vicissitudinem in lacte non inducit sed gelatinam tarde liquefacit. Segregatus e melle et solo. Pro incremento optimo postulat biotinum et acidum pantothenicum sed inositolum necessarium non est.

Acknowledgments

The author is indebted to Mr. John Adams and to Dr. Harold Senn, Division of Botany and Plant Pathology, for the Latin descriptions of the two species.

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THE MIGRATION OF FUNGAL NUCLEI IN AN ELECTRIC FIELD¹

BY E. S. DOWDING² AND E. H. GOWAN³

Abstract

The mycelium of *Neurospora tetrasperma* readily conducts an electric current. During the time an electric current of about 5 μ a. is allowed to pass through cultures of *N. tetrasperma* or *Gelasinospora tetrasperma*, mycelial growth ceases almost entirely, but afterwards the fungi grow normally again and show no ill effects. Electric currents of the order of 1 or 10 μ a. running in either direction through two fused strains of *N. tetrasperma* do not alter the normal direction of nuclear migration from one strain to the other.

Introduction

In a previous paper (6) it has been shown that in the fungus *Gelasinospora tetrasperma* Dowding, after fusion between two strains, *A* and *B*, of opposite sex, nuclei migrate from strain *A* and pass from cell to cell throughout strain *B*, so that strain *B* comes to contain nuclei of both sexes and develops fruiting bodies. This agrees with the discovery of nuclear migration in the Basidiomycetes by Buller (4, pp. 222-223) and in the Ascomycetes by Dodge (5). In *G. tetrasperma* the behaviour of nuclei is as follows: (1) they travel through the plant at a speed of from 4 to 5 mm. per hr. (about twice the growth rate of the hyphae); (2) they pass from cell to cell via the perforations in the transverse septa; (3) although the direction of nuclear migration between paired strains is fairly constant, it can be changed by light; (4) in a growing hypha the cytoplasm streams vigorously towards the tip. Experiment shows that this stream does not carry the nuclei but leaves them behind attached to the thin layer of cytoplasm lining the cell.

Since cytoplasmic streaming is not responsible for nuclear migration, a search for the mechanism was made elsewhere, in the field of electrical phenomena. Taylor and his co-workers (7), using an exceedingly weak direct current through non-polarizable microelectrodes, were able to observe by dark-field illumination the migration of particles through the cytoplasm of a slime mold. In the heterothallic fungi, if it should happen that the (+) and (-) strains possess a naturally different electrical potential, nuclei may migrate from one strain through the other, due to the same cataphoretic force that carries the protoplasmic particles of the slime mold. Experiments were therefore performed to determine whether or not the direction of nuclear migration could be influenced by an electric field.

¹ Manuscript received August 23, 1941.

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Material and Methods

To find suitable material for experiment, a search was made for compatible strains of a fungus whose nuclei under ordinary laboratory conditions and good illumination almost invariably migrate in the same direction. Thus strains "25" and "26" of *G. tetrasperma*, which were first tried, were later rejected because it was found that of 60 paired cultures growing under identical lighting conditions 50 produced perithecia on the "25" side alone, and 10 produced perithecia on the "26" side.

Finally *Neurospora tetrasperma* Shear and Dodge strains "X" and "Y" (of opposite sex), kindly sent by Dr. B. O. Dodge, were tested. About 100 pairings were made, and under the proper moisture conditions perithecia always appeared on the Y mycelium exclusively. These strains of *N. tetrasperma* were therefore used in the experiments on nuclear migration. For a preliminary experiment on the effect of an electric current on fungal development the homothallic *N. tetrasperma* and *G. tetrasperma* were used.

To produce migration of charged particles through a solution by cataphoresis it is necessary for an electric current to flow through the solution. For example, suppose a gum mastic sol is placed in a Petri dish upon the stage of a microscope. Let copper electrodes that dip into the liquid be connected in series with a galvanometer and with a variable source of direct potential. Then as the current is increased from zero, the particles of gum mastic are seen to travel more and more swiftly across the field of view.

To set up an electric experiment with *N. tetrasperma*, a Petri dish of agar was inoculated at opposite ends with the strains X and Y. Near each inoculum, a sterile electrode was bent round the rim of the dish and into the agar. The two inocula were allowed to grow out, and after about 24 hr. the aerial mycelium from each had come into contact with an electrode, and also had approached the other mycelium (Fig. 4). Within another 24 hr. the two mycelia had met and fused. Before the two strains met, a current was sent through the dish and allowed to flow until several hours after they had fused. The current was then disconnected and the cultures were stored for another three days, so that they produced fruiting bodies. The living nucleus could not, of course, be observed under the microscope as were the gum mastic particles, but it was expected that any change in the direction of their migration would show itself later in the distribution of the perithecia. The culture medium used throughout the work was Difco corn meal agar.

In the electrical experiments, it was discovered that when the positive electrode was of copper this substance had a deleterious effect on the development of the fungus. After a current had been run for even a few minutes through such a culture, no perithecia whatever developed on the agar in an area that reached from the positive copper electrode for a radius of about 30 mm. (Fig. 1A). However, transfers from this area to fresh agar showed the fungus to be viable. This effect was due probably to copper diffusion. In later experiments the positive electrode was always platinum.

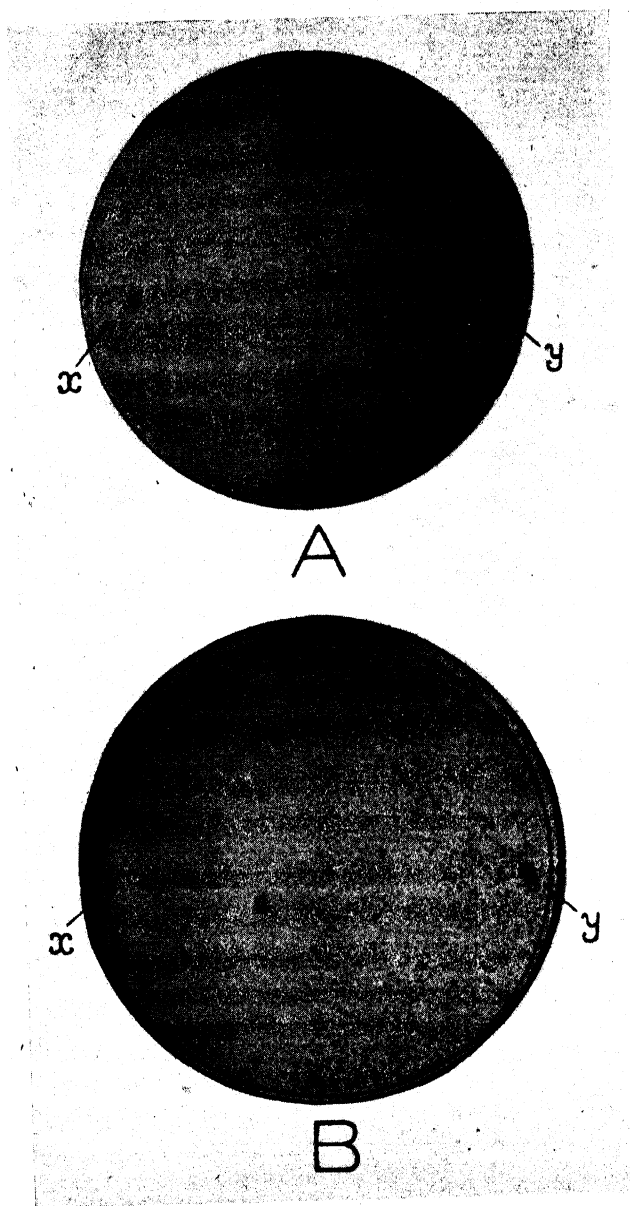


FIG. 1. *Neurospora tetrasperma*, strains X and Y, which were subjected to an electric current during the period of nuclear migration. The normal direction of migration (X nuclei into Y mycelium) was unchanged by the current, so that the Y mycelium bears the perithecia; A, (+) electrode of copper, which has produced an infertile area near it; B, (+) electrode of platinum.

In the electrical experiments, it was found that the two paired mycelia met and that aerial hyphae came into contact with the electrodes at about the same time and then the galvanometer readings gradually increased. When it happened in an experimental plate that no increase in current was recorded during the second day after inoculation, it was assumed that the growing hyphae had not made proper contact with the electrodes, and this plate was not used for experiment.

Occasionally there were sudden changes in galvanometer reading of from 5 to 20 μ a. These jumps were found to be due to faulty contact between electrode and agar, and readings from such cultures were therefore disregarded. In experiments on agar conductivity, the electrodes were firmly secured to the glass dish before the agar was poured in. In Experiment 1, in which the current was run through the agar for a week, the electrodes were made of sheets of lead foil about 2 cm. square and glued to the dish so that the agar as it dried would not shrink from the electrodes.

Five experiments will now be described. The first four are preliminary ones, while the last deals with nuclear migration.

Conductivity Experiments

Experiment 1: Sterile Agar

A Petri dish which had been fitted with electrodes and which was filled with agar, was left on a table where the agar gradually dried at room temperature. The two electrodes were connected with a source of direct current, which gave a constant potential of 1.6 v., and with a galvanometer. The resistance was approximately 30,000 ohms. Galvanometer readings were made one to four times daily for about a week, and are recorded in the following table.

TABLE I
CONDUCTIVITY OF STERILE AGAR DURING DRYING

Elapsed hours	Current, μ a.	Elapsed hours	Current, μ a.
25	18.5	96	17.7
26	17.4	100	17.8
28	16.6	106	17.9
33.5	17.3	120	17.7
47.5	17.4	124	17.7
53	17.6	144.5	17.7
56	17.8	148	17.8
72	17.7	168	17.7
76	17.7		

It may be seen that the conductivity of the agar remained remarkably constant while the experiment was running, the current varying within 1 μ a. from the mean.

This experiment was repeated with two more agar plates and similar results were obtained.

Experiment 2: Agar with Mycelium

A Petri dish fitted with electrodes was filled with agar and inoculated in the centre with a mixture of strains X and Y of *N. tetrasperma*. The electrodes were connected with a source of direct current and a galvanometer. Galvanometer readings were made three or four times daily for three days, that is, during the time the mycelium was growing out over the agar but before it made contact with the electrodes. The average of the 12 readings was $14.7 \mu\text{a}$. and none of the readings varied from this more than $0.8 \mu\text{a}$.

On the fourth day the mycelium had reached the sides of the dish and grown into the air where it came into contact with the electrodes. The galvanometer reading then rose to $17.5 \mu\text{a}$.

Experiment 3: Mycelium Alone

In each of six Petri dishes of agar, two agar strips were cut out so that there would be a double gap in the path of an electric current passing through the plate (Fig. 2). The glass surfaces of the dishes exposed when the agar

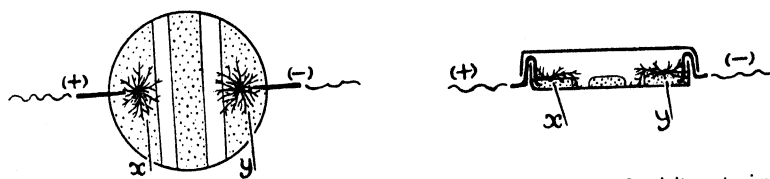


FIG. 2. Surface and side views of *N. tetrasperma* cultures used in conductivity experiment.

was removed were thoroughly dried with sterile cotton wool to reduce possible current leakage to a minimum. Each plate was fitted with electrodes and inoculated with *N. tetrasperma* strains X and Y. The mycelia soon grew out and extended over the glass barriers. A constant potential of 420 v. was applied and galvanometer readings were taken (1) while the paired mycelia were about 1 cm. apart, and (2) after fusion. Table II shows that, in almost all the dishes no current passed until the two mycelia fused, after which the readings ranged from 10 to $52 \mu\text{a}$.

TABLE II
CONDUCTIVITY OF MYCELIUM

Plate number	Current, μa .	
	Before fusion	After fusion
1	0	52
2	4.2	22
3	0	38
4	0	17
5	0	10
6	0	27

The Effect of an Electric Current on Fungal Development

Experiment 4

The normal growth rate of *N. tetrasperma* was first determined as follows. An inoculum was allowed to grow outwards from the centre of the agar in a Petri dish, and then the periphery of the mycelium was traced with ink on the under surface of the dish. During the next eight hours a similar ring was drawn every two hours. By treating several cultures by this method, the growth rate of the mycelium was found to be about 1.5 mm. per hr.

For the electrical experiment, two Petri dish cultures of *N. tetrasperma* (homothallic strain), ringed as before, were placed in a glass-topped box containing a saturated atmosphere. Platinum wires dipped into the agar served as negative electrodes. The positive electrodes were sharpened copper wires fixed about 2 cm. above the mycelium (Fig. 3). The lid of one culture was removed, allowing the current to pass through the mycelium. The lid of the second (control) culture was left on, screening the mycelium from the current. A potential of 5000 volts was applied to the electrodes.

The current through the mycelium was calculated as follows. The leakage current around the box was $0.6 \mu\text{a.}$; the current through the electrode above the covered dish was $2.1 \mu\text{a.}$; that through the electrode above the open dish was $4.7 \mu\text{a.}$ Thus about $2 \mu\text{a.}$ went through the tips of the aerial hyphae to the agar and thence to the negative electrode.

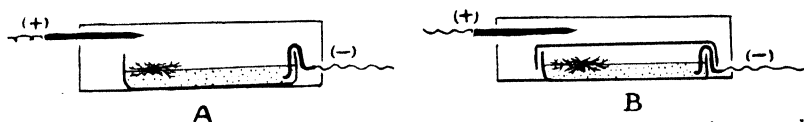


FIG. 3. Cultures of *N. tetrasperma* used in electric current experiment; A, exposed culture; B, control.

In order to calculate approximately the total cross sectional area of fungus through which the current passed, the procedure was as follows. An inoculum of *N. tetrasperma* placed at one end of a Petri dish of agar, was allowed to grow out over a film of water spread over a microscopic slide which just fitted inside the dish and lay on the agar. After a sheet of mycelium had covered the slide it was cut away from the agar and gathered into a roll of a length equal to the width of the slide. The diameter of the cylinder of mycelium was 1 mm., which is only 5% greater than the effective diameter, i.e., the diameter of a solid cylinder having the same cross sectional area as the actual cylindrical bundle of hyphae, the individual threads in the bundle being supposed for the purposes of calculation to be circular in section and packed as tightly as possible. A current of $2 \mu\text{a.}$ had passed through the cylinder; this represents about $2.6 \mu\text{a. per mm.}^2$ of mycelium.

After the current had run for four hours it was disconnected and the cultures were ringed again. It was found that the growth of the mycelium in the experimental plate during the four hours was practically negligible,

while the control had grown 7 mm. (about the normal rate). The growth of the experimental culture was measured again for several hours after the current had been disconnected. It was found that normal growth rate was resumed immediately after the current had ceased to flow, and in a few days perithecia formed normally.

A similar experiment was carried out using *G. tetrasperma* and similar results were obtained.

The conclusion is that a current of about $2\frac{1}{2}$ μ a. per mm.² through the mycelium retards growth while it is flowing, but has no permanently injurious effect either on growth or reproduction.

In Experiment 5 on nuclear migration the current was run after growth had ceased. Although the current that passed through the mycelium was probably 10 times greater than that which in Experiment 4 had been found to arrest growth, the fungus suffered no ill effects and produced perithecia normally.

Blackman and Legg (1) working with pot cultures of cereal seedlings, found that an electric current of the order of 0.01 μ a. per plant retarded growth. The barley coleoptiles which they used measured about 0.013 cm.² in cross section (2), so that the current per mm.² must have been 0.008 μ a., or about 1/300 of the current in this experiment.

Discussion

In the past, numerous experiments have been carried out to determine whether or not the growth and yield of a field of crop plants could be increased by the presence of an electrically charged network over it. Such "Electroculture" experiments carried out in England showed a marked increase in yield, but in the United States similar experiments did not confirm this finding (3). It would be a comparatively simple undertaking to extend the electrocultural investigations by experiments (similar to the one described above) on fungi such as *Neurospora* and *Gelasinospora*, which are small and easy to culture, and complete their life history in less than a week.

The Effect of an Electric Field on Nuclear Migration

Experiment 5

Fourteen Petri dishes were inoculated at opposite ends with *N. tetrasperma* strains X and Y. Eight of the plates served as controls, while the remaining six were fitted with electrodes and used for experiment as in Fig. 4. In

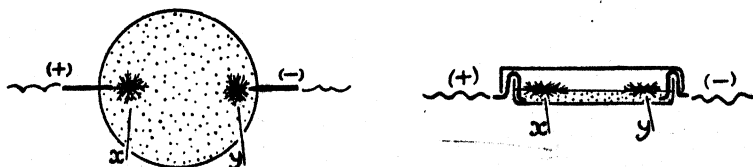


FIG. 4. Surface and side views of *N. tetrasperma* cultures used in electric field experiment.

one-half of the experimental plates the *X* inoculum was near the (+) electrode and in the other half it was near the (−) electrode. To eliminate light as a cause of any possible change in the direction of nuclear migration, the *Y* mycelium, into which *X* nuclei normally migrate, was always placed nearest the light.

The day following inoculation the electrodes were connected with a 420 v. power supply and a galvanometer, the cultures being connected in series. At this time the current through each Petri dish was 11 μ a. Current readings taken at intervals, from just before fusion until shortly after fusion was complete in all the plates, gradually increased from 13.7 to 22 μ a., as shown in Table III.

TABLE III
CONDUCTIVITY OF SIX CULTURES DURING PERIOD OF FUSION

Elapsed hours	Current, μ a.	Remarks
18	13.7	Mycelia not in contact
22	14.5	One pair of mycelia in contact
24	18	All pairs in contact
27	19.3	Fusion probably commencing
29	22	Fusion probably complete
31	22	No change

After the current had been running for two days it was disconnected, and the cultures were stored for another five days, by which time they had produced perithecia.

In the eight control and the six experimental plates the perithecia had developed on the *Y* mycelium exclusively (Fig. 1B). Nuclei must have migrated always from the *X* to the *Y* mycelium, irrespective of the direction of the electric current.

A second experiment was carried out, this time using a lower potential so that the current per plate was of the order of 1 μ a. Six cultures were used. In three plates the current travelled in one direction, and in the other three in the opposite direction. Galvanometer readings during fusion rose from 0.6 to 0.9 μ a. When the cultures produced perithecia their distribution (on the *Y* mycelium) showed that the current had caused no change in the direction of nuclear migration in any of them.

Discussion

The experiments of running an electric current in either direction through fusing strains of *N. tetrasperma* produce no change in the normal direction of nuclear migration. Yet it cannot be assumed from this that cataphoresis is not the mechanism of nuclear migration. It is impossible to say whether or not under the conditions of the experiments the fungal cytoplasm was actually in an electric field. It is possible that the electric current, instead

of travelling through the cytoplasm, passed along the cell walls of the hyphae or even along the film of water condensed upon them. Even supposing that the current passed through the cytoplasm, the amount of current used (1 and 10 μ a.) may not have been the effective strength for cataphoresis of nuclei.

Again, since it is already known that light readily influences the direction of nuclear migration in *Gelasinospora* (6), it may be that *Gelasinospora* would have been more suitable than *Neurospora* for electrical experiments. However, since *Gelasinospora* grows best in very humid conditions, there would be the difficulty of leakage currents through water films to be overcome. Further, since the direction of nuclear migration in *Gelasinospora* is normally inconstant, many more experiments and controls would be needed than in *Neurospora* to establish the significant effect of an electric field.

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BIOLOGICAL DECOMPOSITION OF CHEMICAL LIGNIN

III. APPLICATION OF A NEW ULTRA-VIOLET SPECTROGRAPHIC METHOD TO THE ESTIMATION OF SODIUM LIGNOSULPHONATE IN CULTURE MEDIA¹

By G. A. ADAMS² AND G. A. LEDINGHAM³

Abstract

An ultra-violet spectrographic method has been developed for estimating the amount of sodium lignosulphonate present in solutions. The method has been applied to the measurement of lignosulphonate losses in liquid media after fungous growth and the results are compared with those obtained under similar conditions by the β -naphthylamine precipitation method. The spectrographic method gave slightly lower values but was free from certain errors present in the chemical method. The results confirmed previous observations that fungi are capable of decomposing lignosulphonates.

The quantitative estimation of lignosulphonates presents several difficulties. Since they do not crystallize, they cannot be separated and directly weighed, neither are there characteristic groups occurring in them in constant amounts which can be used as a basis for estimation. Attempts to use the methoxyl group have not been entirely satisfactory. The methods of estimation hitherto used most frequently have been by precipitation, using 80% ethanol or methanol, calcium oxide, lead acetate, sodium chloride, calcium chloride, sulphuric acid, or β -naphthylamine. The most efficient of these has been found to be β -naphthylamine (7).

In the course of a study on the decomposition of lignosulphonate solutions by fungi, it was necessary to measure accurately the lignosulphonate in the culture media before and after fermentation. These measurements were made by a method employing β -naphthylamine precipitation (1). However, incomplete precipitation leaves room for inaccuracies, especially in media where fungi have been growing and consequently have brought about changes in the medium as compared with the uninoculated controls. In experiments with wood destroying fungi there were instances where the β -naphthylamine precipitate actually weighed more than that of the controls (6).

In searching for a more reliable and accurate method for determining sodium lignosulphonate, a method was developed using quantitative measurements of the absorption spectrum of sodium lignosulphonate in the ultra-violet region. The ultra-violet spectrum of lignin and its derivatives has been studied extensively (2, 3, 4, 8) and it has been established that the lignin derivatives of softwoods have a band in the ultra-violet with the maximum between 2800 Å and 2870 Å. Cellulose and other carbohydrates do not exhibit any light absorption in this ultra-violet region (5). Conse-

¹ Manuscript received September 5, 1941.
Contribution from the Division of Biology and Agriculture, National Research Laboratories, Ottawa. Issued as N.R.C. No. 1024.

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quently it is possible to measure the ultra-violet absorption spectrum of lignin even in the presence of carbohydrates. The absorption in the region of 2800 Å appears to be characteristic of a main molecular structure and not of some specific groups, and a quantitative measurement of the light absorption can be interpreted in terms of the amount of liginosulphonate in the solution.

Apparatus

The absorption spectra were determined photographically using a Hilger medium quartz spectrograph with a Hilger Spekker ultra-violet photometer. The slit width was set at 0.03 mm. The light source was a spark between tungsten steel electrodes supplied by an oscillating current from 0.25 mf. and 2 mh. fed by a d-c. rectifier giving 18 ma. at 12,000 v.

Eastman No. 40 spectrum analysis plates, developed in Eastman D72, were used throughout, as their high contrast made the picking out of points of equal intensity a relatively easy task.

Cells 2 cm. long were used for holding the solution and solvent. These were fitted with quartz windows at each end, held in place by a screw cap.

Technique

Exposures were taken at varying apertures as read by the density drum calibrated in 0 to 1.5 density units. The time of exposure was varied with the drum reading. About a three-second exposure was sufficient for a density reading of zero.

One cell was filled with control solution and the other with control solution to which sodium liginosulphonate had been added. The spectrograms of the two solutions were taken simultaneously so that minor variations in the light source had no effect on the results.

Development of Method

Spectrum Characteristics

It was first necessary to determine whether the sodium liginosulphonate had a spectrum characteristic of lignin derivatives, and whether the nutrients used along with the sodium liginosulphonate in the culture media had spectra that would mask or interfere with the liginosulphonate spectrum. Spectra were taken of a series of varying concentrations of sodium liginosulphonate in water. It was found that a concentration of 0.005% was most suitable for this study. The spectrograms showed sodium liginosulphonate to have a well defined absorption band with a maximum absorption at 2800 Å corresponding to the spectrum of lignin derivatives in general.

Preparation of the Solutions

A number of the common nutrients used for fungal growth were next tested to find if their ultra-violet spectra showed absorption in the same region as the lignin compounds. The solutions tested were 1% ammonium nitrate, 1% potassium acid phosphate, 0.5% magnesium sulphate, 1%

potassium chloride, 1% dibasic ammonium phosphate, 1% ammonium carbonate, trace of ferrous sulphate, and 3% glucose. Sodium lignosulphonate was run on the same plate to facilitate easy comparison. The plates were taken with three seconds' exposure, density zero. It was found that ferrous sulphate was slightly absorbing and ammonium nitrate heavily absorbent, so fungi that grew well without iron in the medium and that did not require ammonium nitrate as a source of nitrogen had to be selected. It was now possible to select from the remainder, nutrients that would make a satisfactory control. A suitable culture medium was made up containing:—

Dextrose	20.0 gm.
Dibasic ammonium phosphate	5.0 gm.
Magnesium sulphate	1.0 gm.
Potassium chloride	1.0 gm.
Sodium lignosulphonate	37.5 gm.
Water	1000 cc.

Another spectrographic control solution was made up which contained all the above constituents except lignosulphonate. The two solutions were sterilized with steam for 15 min. at 15 lb. pressure. The control solution turned a light brown colour during sterilization. To demonstrate that the colour change does not affect the spectrum in the band studied, the following check was made. Both solutions were diluted equally, the sodium lignosulphonate concentration in the one being 0.005%. Spectrograms of the following solutions were then taken with zero density and three-second exposure: (1) the sterilized nutrients against water; (2) the sterilized nutrients against sterilized media. Using water as a check, it was demonstrated that the sterilized nutrients have no absorption band in the region at 2800 Å, while the sterilized medium has a pronounced absorption. This shows that the absorption in the medium is due entirely to the sodium lignosulphonate and not to the other nutrients.

Preparation of Standards

In order to convert the spectrographic data obtained from the spectra of the lignosulphonate culture medium into actual amounts of lignosulphonate existing in the medium, it was necessary to have a set of standards prepared in which the density reading (D) at the wave length of maximum absorption was known for a series of various concentrations of lignosulphonate. A standard culture medium containing 37.5 gm. of sodium lignosulphonate diluted with water to give a 0.005% solution was used. This was photographed in the ultra-violet, along with a similar solution with no lignosulphonate as control. This original solution was diluted to produce sodium lignosulphonate contents of 95, 90, 85, 80%, respectively, on which densities were determined as shown in Fig. 1.

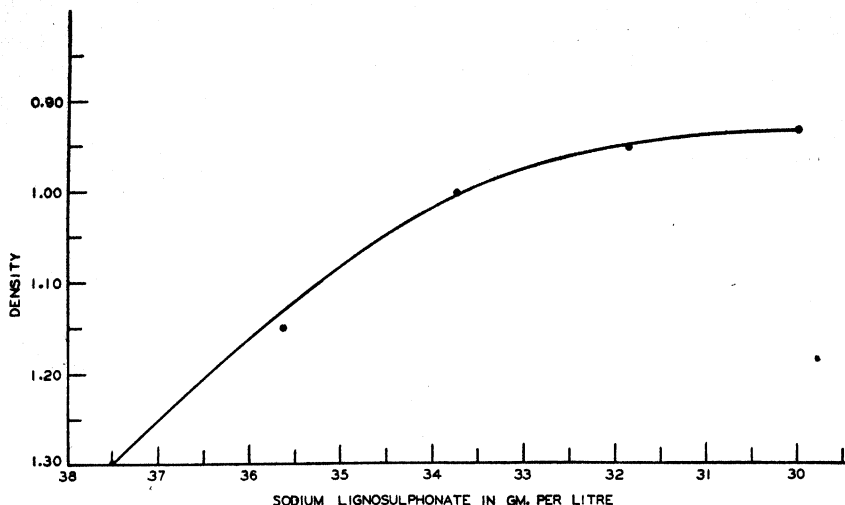


FIG. 1. Graph relating concentration of sodium lignosulphonate to density.

Method

The culture medium was made up, sterilized, and pipetted in 100 ml. lots into six flasks of 500 ml. capacity. Three of the flasks were inoculated with spore suspensions of the fungus and three were not inoculated but kept as controls to find whether or not the medium underwent changes without microbial growth. The cultures and controls were incubated in the dark at 25° C. for three weeks.

At the end of the growth period the fungus mat was filtered from the solution and washed thoroughly with water until all traces of the medium had been removed. The washings were added to the original filtrate and the whole made up to a volume of 200 ml. From this solution 0.66 ml. were diluted to 250 ml. for spectrographic analysis. A control was made up from the sterilized nutrient salts without the sodium lignosulphonate.

The spectrograms were taken using 21 density readings from 0.70 to 1.40 with exposure times varied from 10 sec. for density 0.70 to 44 sec. for density 1.40. These densities gave a sufficiently well defined absorption curve to facilitate selection of the point of maximum absorption. The density reading at the peak of the absorption was then transposed to the graph (Fig. 1) relating density and concentration of sodium lignosulphonate and the amount of the lignosulphonate in the solution was read off directly. Since the amount in the original culture medium was known, the loss due to decomposition was readily calculated.

Application of the Method

The culture medium on which *Fusarium culmorum* (W. G. Smith) Sacc. had been grown was analysed at the end of 20 days by the β -naphthylamine

precipitation method and by the spectrographic method described above. The loss in lignin shown by the two methods was as follows:—

Precipitation method	7.82%
Spectrographic method	6.94%

A spectrographic examination of the uninoculated culture medium after a period of three weeks indicated that it had remained unchanged in so far as the spectrum of the sodium lignosulphonate was concerned.

Another experiment was carried out in which *Alternaria* sp. (T-S-29) was used for the breakdown of the sodium lignosulphonate. The organism was grown for a period of three weeks and produced a thick heavy mat. The loss in lignin from this culture as found by the two methods of analysis is as follows:—

Precipitation method	6.42%
Spectrographic method	5.02%

Analytical Results on Wood Destroying Cultures

It has been previously pointed out that culture media on which wood destroying fungi have been grown, when treated with β -naphthylamine reagent to precipitate the lignosulphonate, form a precipitate which is not a hard, dark plastic as in the control, but is a coarse dark red powder (6). This type of precipitate weighs more than its corresponding control from an uninoculated culture and gives the apparent result of the lignin concentration being increased during the growth of the fungus. It seemed likely that a spectrographic investigation of this problem would throw more light on the fate of the lignosulphonate during the growth period.

The culture medium was prepared in the same way as outlined previously. The wood destroying organisms, *Poria subacida* (Peck) Sacc. and *Polyporus dichrous* Fr., were grown in duplicate with two uninoculated control flasks. The analyses were carried out at 47 days when *Poria subacida* showed small scattered colonies in all duplicate cultures while *Polyporus dichrous* had heavy submerged spongy mats. The lignin content was determined both by the β -naphthylamine precipitation method and the spectrographic method, and the results are shown in Table I.

TABLE I
RESULTS OF SODIUM LIGNOSULPHONATE ANALYSES ON MEDIA AFTER GROWTH
OF WOOD DESTROYING FUNGI

Sample	Lignosulphonate decomposed, %	
	Chemical method	Spectrographic method
<i>Poria subacida</i> (1)	(Gained in wt.) 23.12	2.93
<i>Poria subacida</i> (2)	(Gained in wt.) 17.71	4.53
<i>Polyporus dichrous</i> (1)	2.19	4.53
<i>Polyporus dichrous</i> (2)	0.31	4.53

It can be seen from the results in Table I that when the lignosulphonate is determined by the chemical method for *Poria subacida* cultures, there is a marked increase in weight of the precipitate over the uninoculated control, thus giving an apparent increase in the lignosulphonate of the medium. On the other hand the spectrographic results, which are free from the errors of the chemical method, indicate small but positive decomposition of the lignosulphonate. It is apparent that some product of metabolism formed in the growth of the fungus is precipitated with the lignosulphonate or else some change has taken place in the medium that renders more of the lignin precipitable by β -naphthylamine, 68% normally being precipitable.

Although *Polyporus dichrous* cultures showed a positive loss in lignosulphonate when analysed by both methods, the spectrographic results were considerably higher. The fact that the nature of β -naphthylamine precipitate from these cultures was beginning to change to the same type as found in the cultures of *Poria subacida* indicates that the same phenomenon is taking place but has not progressed so far. If the growth period had been prolonged, an apparent increase in lignosulphonate would have occurred. This indicates

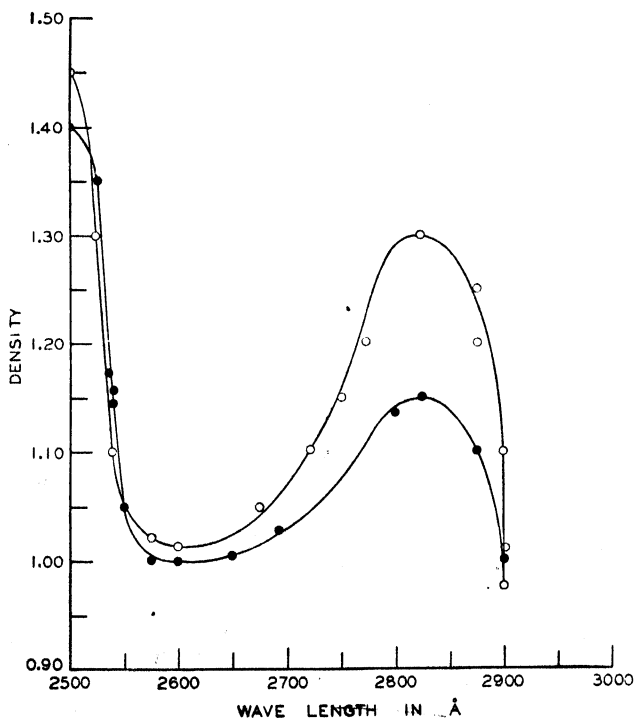


FIG. 2. Absorption spectra curves of sodium lignosulphonate. —○—○— Uninoculated solution. —●—●— Solution on which *Fusarium culmorum* had been grown for 20 days.

that the precipitation method does not give a true measurement of lignosulphonate decomposition, especially in cultures that have been growing for a long period of time.

Discussion

The absorption spectra of almost all lignins that have been studied show an absorption band at about 2800 Å. It has been possible to make use of this specific absorption in solutions of sodium lignosulphonate to measure the amount of the substance present before and after breakdown by micro-organisms. The form of the absorption curve from the inoculated cultures is in strict conformation to that of the uninoculated control solution, although the amount of absorption varies as shown in Fig. 2. Apparently the lignosulphonate that has been broken down by the organism has been changed into a substance or substances that have no interfering absorption in this region of the ultra-violet. It appears likely, therefore, that the molecule of sodium lignosulphonate has been wholly converted to some simpler molecule, rather than partially modified into another related substance. If it were changed slightly in its chemical composition, the absorption curve would not be expected to remain identical in form with that of the uninoculated lignosulphonate medium. Examination of uninoculated lignosulphonate culture medium shows that it does not undergo any changes in so far as its spectral properties are concerned, even in 50 days, provided it is stored in the dark.

Acknowledgments

The authors wish to acknowledge the assistance of Dr. C. A. Horton, of the Division of Physics, for valuable aid in the spectrographic phases of this problem, and also the technical help of Mr. A. Castagne.

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RUST REACTIONS OF CHINESE WHEAT VARIETIES AND CERTAIN CANADIAN HYBRID STRAINS¹

BY YUN-CHANG WANG²

Abstract

About 160 Chinese spring wheats and a number of Chinese winter wheats were tested in the seedling stage in the greenhouse to determine their reaction to eight physiologic races of stem rust (*Puccinia graminis Tritici*) and to four physiologic races of leaf rust (*Puccinia triticina*). The spring wheats were also subjected, in the field, to epidemics of stem rust and leaf rust in which a large number of physiologic races of each rust were employed. The wheats were classified as one or another of the following species: *Triticum vulgare*, *T. compactum*, *T. durum*, and *T. turgidum*.

By means of these tests, it has been demonstrated that the Chinese wheats are rather highly susceptible to the physiologic races of stem rust prevalent in North America. Many of the wheats showed, however, considerable resistance to leaf rust in both the greenhouse and the field tests. Varieties resistant to leaf rust were present in all of the four above-mentioned species.

Seventy-five lines of Canadian spring wheat derived from a cross between Renown Selection, which is resistant to stem rust and moderately resistant to leaf rust, and Garnet, which in North America is resistant to stripe rust (*Puccinia glumarum*), were tested in the seedling stage in the greenhouse for their reaction to stripe rust. Two physiologic races (race 6 and race 13) were used in these tests. The same lines were subjected to rust epidemics in the field to determine their resistance to stem rust and leaf rust. Several of these lines were found to possess resistance to all three rusts. These lines may prove valuable breeding material in case it is desired to develop by breeding methods Chinese varieties resistant to all three of these rusts.

Introduction

No reliable record exists of the losses caused by cereal rusts in China, but there can be no doubt that these are often severe. In the northern part of Honan, in north China, the writer has noticed that, in years of abundant moisture and heavy stand of grain, the damage caused by rust was particularly severe. In certain years, when normal yield was expected, he has seen an outbreak of rust reduce the yield to 20 or 30% of that anticipated. Farmers in that area consider this disease as the worst of all the troubles of the wheat crop, with the possible exception of drought, which occurs frequently in north China.

Wheat, in China, is parasitized by three separate species of rust, namely, stem rust (*Puccinia graminis Tritici* Erikss. & Henn.), leaf rust (*Puccinia triticina* Erikss.), and stripe rust (*Puccinia glumarum* Erikss. & Henn.). As far as the writer has been able to discover, the two last-mentioned rusts cause the most serious damage.

¹ Manuscript received August 26, 1941.

Contribution No. 681, from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada.

² The work was carried out at the Dominion Laboratory of Plant Pathology, Winnipeg, Man., during the author's tenure (1938-1939) of a fellowship granted him by the China Foundation for the Promotion of Education and Culture.

The present work was undertaken as a result of the writer's realization of the severity of the losses caused by these rusts and the necessity of breeding varieties resistant to them. Such a project can only be accomplished by utilizing and applying the principles of plant breeding and plant pathology, as has been done with conspicuous success in North America and elsewhere, through the close co-operation of plant breeders and plant pathologists. In the present work, the writer has attempted to discover the relative resistance of different wheat varieties that might be of value for a breeding program. In an attempt to attain this object two different methods have been pursued. The first was to try to find the necessary rust resistance among Chinese wheat varieties. This has been attempted by testing a large number of Chinese wheats for their resistance in the greenhouse and in the field to many physiologic races of leaf rust and stem rust. In this work, it was unfortunately not possible to test the resistance of the wheat varieties to stripe rust as this rust does not occur in the central part of North America, where the work was conducted, and was not available for greenhouse studies during most of the period when the work was in progress. The second method employed was to try to discover, among foreign wheats, varieties that possess resistance to the three above-mentioned rusts. Such varieties would be useful as breeding material even if they were not themselves of value under conditions in China. In this category may be included certain lines recently developed at the Dominion Rust Research Laboratory from a cross between Garnet, which in North America is resistant to stripe rust (6), and Renown, which is resistant to stem rust and fairly resistant to leaf rust (5, 7). If some of these lines were found to be resistant to all three rusts, they would be extremely useful for purposes of crossing with Chinese wheat varieties. For this reason greenhouse tests were conducted with 75 of these lines to determine their resistance to stripe rust, and field experiments were carried out to test their resistance to stem rust and leaf rust.

The problem of determining the resistance of any wheat variety to stem rust, leaf rust, or stripe rust is complicated by the fact that each of these rusts is not a unit, but consists of many different strains known as physiologic races. To determine the resistance of any variety to one of these rusts, it is necessary to test its resistance not only to one but to many physiologic races. In the present work, it would have been desirable to test the resistance of the Chinese wheats to physiologic races collected in China. This, however, was not possible. In the absence of rust from China, the varieties were tested for their resistance to several physiologic races that occur commonly in North America. Two of the races of stem rust used in these tests (races 9 and 15) have been reported by Tu (10) as occurring in China. Probably these tests furnish an indication of the resistance of wheats to the stem rust races that occur in China, although only six races so far seem to have been identified in that country.

Materials and Methods

About 200 Chinese wheats were tested in the greenhouse and in the field to determine their resistance to stem rust and leaf rust. These wheats were obtained by the writer from the United States Department of Agriculture through the kindness of Dr. S. C. Salmon, agronomist in charge of wheat investigations. They were originally obtained, for the most part, from the University of Nanking, Nanking, China, and represent nearly all the wheat growing regions of China. These wheats, with a few exceptions, were of spring habit.

Owing to the fact that identification of wheat varieties can be made with certainty only in the adult stage of growth, it was possible to identify only the varieties that were grown to maturity in the field. Among the varieties that could not be identified are all the winter wheats, and certain spring wheats of which the quantity of seed received was only sufficient for seedling tests. The great majority of the Chinese wheats studied were identified as one or another of the four species, *Triticum vulgare* Host, *T. turgidum* L., *T. durum* Desf., and *T. compactum* Host.

The bulk of the Chinese wheats, 165 samples, were identified as common wheat, *T. vulgare*. These had their origin in nearly every province in China.

The rivet wheats, *T. turgidum*, in this collection, were limited to two samples, one from Kansu and one from Sinkiang (Chinese Turkestan).

Eight of the 14 specimens of *durum* wheat, *T. durum*, originated from Yunnan, the remainder, from Kansu, Szechwan, Honan, and Sinkiang.

There were also included in this collection 14 club wheats, *T. compactum*, eight from Szechwan, three from Kansu, and three from Yunnan.

Two different kinds of tests were employed to determine the resistance of the Chinese wheats to stem rust and leaf rust: (1) greenhouse tests in which known physiologic races were employed, and (2) field tests in which the wheats were subjected to natural rust infection plus artificially induced epidemics of both rusts. In the greenhouse tests, varietal resistance was judged by the reaction to rust of seedling leaves inoculated with pure cultures of physiologic races. For the tests with stem rust, eight physiologic races were employed, namely, races 9, 15, 19, 21, 34, 38, 51, and 56. In the tests with leaf rust, races 1, 9, 58, and 76 were used. The field experiment comprised separate tests for each of the two rusts, the varieties, in both tests, being planted in five-foot rows surrounded by border rows of the susceptible wheat Little Club. On these border rows an epidemic was induced artificially by dusting spores of many physiologic races over the plants by the use of a duster made specially for this purpose.

For the test with stripe rust, seed of 75 lines of Canadian wheat was obtained from Dr. R. F. Peterson, of the Dominion Rust Research Laboratory, Winnipeg, Man. These were F_4 lines from a cross between Renown Selection (R.L. 716.6) and Garnet. Of these parents the former is resistant to both stem rust and leaf rust while the latter has considerable resistance to stripe

rust and matures very early. The tests of the stripe rust reaction of these hybrid lines were conducted in the greenhouse by inoculation of seedlings with physiologic races 6 and 13.

The commonly accepted system of recording infection types of cereal rust on seedling plants was adopted in recording the different rust reactions in the greenhouse tests. For stem rust of wheat, the system of Stakman and Levine (9) was used; for leaf rust of wheat, that of Mains and Jackson (4); and for stripe rust that of Hungerford and Owens (2).

For recording the severity of the infection on the adult plants, the scale given by Clark, Martin, and Stakman (1) of the Office of Cereal Crops Diseases, United States Department of Agriculture, was used. According to this scale, rust severity is recorded as varying from a trace to 100%. Those plants with very few pustules are recorded as having a trace of rust and those with maximum infections as having 100% severity.

Reaction of Chinese Wheats to Stem Rust and Leaf Rust

The results of the tests conducted in the greenhouse and in the field to determine the reaction of Chinese wheats to stem rust and leaf rust are summarized in Table I.

The tests of the reaction of 184 Chinese wheats to the eight physiologic races of stem rust (races 9, 15, 19, 21, 34, 38, 51, and 56) showed that not one of these wheats possessed resistance in the seedling stage to all eight races, although a number of wheats were resistant to one or more of the races. Among wheats of the *vulgare* type, no variety was found to be resistant to more than three of the eight races. In *T. durum* and in the winter wheats, the proportion of varieties possessing some resistance was greater than in the *vulgare* wheats of spring habit. The lack of stem rust resistance in the Chinese wheats was confirmed by the field test in which all of the 189 wheats tested proved susceptible.

Considerably more resistance was shown by the Chinese wheats to physiologic races of leaf rust. In *T. vulgare* several varieties of diverse morphologic types showed resistance in the seedling stage to the four races employed in the greenhouse test (races 1, 9, 58, and 76). Most of the wheats resistant in the greenhouse tests were also resistant in the field test, but several wheats that had proved susceptible in the seedling stage showed resistance in the field. Consequently the proportion of resistant wheats was markedly larger in the field test. This disparity between the two tests would indicate the presence among some of the Chinese varieties of mature plant resistance to leaf rust.

Reactions of Certain Canadian Spring Wheats in the Seedling Stage to Two Physiologic Races of *Puccinia glumarum*

It would have been desirable to determine the reactions of the Chinese wheats to stripe rust (*P. glumarum*) as well as to stem rust and leaf rust. As already stated, this was not possible as stripe rust was not available

TABLE I
REACTION, IN THE GREENHOUSE AND IN THE FIELD, OF CHINESE WHEATS TO STEM RUST
AND LEAF RUST

Species of wheat tested	Greenhouse reaction							
	Stem rust				Leaf rust			
	Number wheats tested	Number S or MS to all eight races	Number R or MR to one or more races	Number R or MR to all eight races	Number wheats tested	Number S or MS to all four races	Number R or MR to one or more races	Number R or MR to all four races
Spring wheats								
<i>T. vulgare</i>	142	124	18	0	138	98	31	9
<i>T. compactum</i>	10	8	2	0	10	3	7	0
<i>T. durum</i>	8	5	3	0	7	6	0	1
<i>T. turgidum</i>	1	1	0	0	1	0	1	0
<i>T. spp.</i>	2	1	1	0	1	1	0	0
Winter wheats	21	12	9	0	23	14	8	1
Total	184	151	33	0	180	122	47	11
Species of wheat tested	Field reaction (Natural and artificially induced epidemic)							
	Stem rust				Leaf rust			
	Number wheats tested	S	MR	R	Number wheats tested	S or MS	MR	R
Spring wheats								
<i>T. vulgare</i>	157	157	0	0	162	81	39	42
<i>T. compactum</i>	16	16	0	0	13	2	1	10
<i>T. durum</i>	14	14	0	0	12	0	8	4
<i>T. turgidum</i>	2	2	0	0	2	0	0	2
<i>T. spp.</i>	—	—	—	—	—	—	—	—
Winter wheats	—	—	—	—	—	—	—	—
Total	189	189	0	0	189	83	48	58

Explanation of symbols:—

S=susceptible; MS=moderately susceptible; MR=moderately resistant; R=resistant.

during most of the period of the greenhouse tests. In the spring of 1939, two physiologic races of stripe rust (races 6 and 13) were brought into culture in the greenhouse. At this time, it was too late to commence tests with the Chinese wheats.

As certain Canadian wheats, which were known to be resistant to stem rust and leaf rust, were also thought to possess resistance to stripe rust, the writer decided to test these for their resistance to the two races of stripe rust, because any of these strains found resistant to all three rusts would be of great value in any Chinese plant breeding program designed to develop rust resistant wheats. Seventy-five such lines were included in the test. These

lines were derived from F_3 plants, which appeared to be resistant to stem rust and leaf rust in field tests in 1938. All of the lines were tested for their reaction to race 6, but, owing to shortage of inoculum, it was possible to test only 25 of the lines for their reactions to race 13. Of these 25 lines, five were resistant to both races, seven were resistant to race 13 and moderately resistant to race 6, while one line was moderately resistant to both races. Of the remaining 50 lines which were tested to race 6 alone, 20 showed immunity or resistance. As these lines are resistant to stem rust and as most of them proved resistant to leaf rust in a field test conducted in the summer of 1939, they would appear to hold considerable promise as breeding material.

Discussion

Although wheat has been cultivated in China for a very long time, it is only in recent years that scientific attention has been given to the botanical or agronomic characteristics of Chinese wheats or to the diseases to which they are subject. Of these diseases the rusts are perhaps the most important. The desirability of developing wheat varieties resistant to rusts can therefore not be questioned. As all three of the rusts of wheat, stem rust, leaf rust, and stripe rust, occur in China, the problem of developing rust resistant wheats becomes one of combining in a single variety resistance to all of the three rusts.

It seems clear that the first step towards the attainment of this objective is to discover to what extent existing Chinese varieties are resistant to these rusts. The present work has been largely devoted to this task. In this work, about 200 Chinese wheats have been tested for their resistance to some of the most prevalent physiologic races of stem rust and leaf rust in North America. For reasons stated elsewhere in this paper it was not possible to test the resistance of these varieties to stripe rust.

No country that has attempted to solve its rust problems by plant breeding methods depends, however, entirely on its own varieties. On the contrary, wherever large-scale plant breeding programs have been undertaken, varieties with the desired qualities have been gathered together with the purpose of combining these qualities with those of varieties of local origin in an attempt to create new varieties suitable to the particular climatic and soil conditions of any given country. Any plant breeding program organized in China must therefore be based on the best available genetic material, whether this be of local or of foreign origin. It was with this idea in mind that the present work was extended to include tests of the resistance to stripe rust of certain Canadian spring wheat lines known to possess resistance to stem rust and leaf rust and thought to possess also resistance to stripe rust.

The development of these lines is worth brief consideration as it illustrates the methods used by Canadian plant breeders in combining in one variety resistance to several rusts. The lines originated in a cross between Renown and Garnet. In Renown is combined (in the adult stage of growth) high resistance to stem rust and a considerable resistance to leaf rust (5, 7).

Garnet, while susceptible to stem rust and leaf rust (5, 7), is resistant to stripe rust (3, 6, 8). In the F_2 and F_3 generations of the cross, the lines resistant to stem rust and leaf rust were selected but, as stripe rust does not occur in Manitoba, where the work was done, no selection could be made for resistance to that rust. As it was thought probable that some of the F_4 lines would resemble the Garnet parent by being resistant to stripe rust, the writer tested in the greenhouse the resistance of these lines to two physiologic races of stripe rust. Any lines that were resistant to all three rusts would be of considerable value as breeding material, in case Chinese wheats are susceptible to this rust.

The task of producing rust resistant wheats is complicated by the existence of many physiologic races of each of the cereal rusts. Little is known, as yet, about the specialization of these rusts in China. Tu (10), however, has shown that several physiologic races of stem rust of wheat are present, among which are included some races known to occur in America. In the present work, physiologic races collected in Canada were used for testing the Chinese varieties. Although such tests with rust of foreign origin may be of value in indicating which varieties possess rust resistance, a decision as to the suitability of the varieties for plant breeding purposes in China must depend on their resistance to rust races of Chinese origin.

As far as it is possible to judge from tests with rusts that are not of Chinese origin, it would appear that Chinese wheats are highly susceptible to stem rust but possess considerable resistance to leaf rust. It follows that, if Chinese varieties resistant to both these rusts are to be developed, the resistance to stem rust may have to be introduced from foreign varieties. It is possible, though by no means certain, that the so-called "mature plant resistance", which forms the basis of resistance to stem rust in most of the new American and Canadian spring wheats, may also be of value as a basis for resistance to rust in the spring wheat areas of China. The chief value of this type of resistance lies in the fact that varieties possessing it acquire, as they grow towards maturity, a resistance to all physiologic races, irrespective of how susceptible in the seedling stage they may be to the same races. If mature plant resistance should prove equally effective in China as in North America, it should not be difficult, by means of crosses, to combine it with the leaf rust resistance of Chinese wheats and other desirable qualities possessed by them.

Although nothing is known to the writer concerning the reaction of Chinese wheats to stripe rust, it appears that the course of action outlined above is also applicable to the production of varieties resistant to this rust. If the Chinese varieties should prove resistant to stripe rust, it will not be necessary to seek resistance to this rust from some other source. If they should prove susceptible, resistance to this rust must be sought elsewhere. It was with this possibility in view that the writer attempted to investigate the stripe rust resistance of Canadian spring wheats that already had the merit of resistance to stem rust and leaf rust.

Acknowledgments

The writer wishes to make grateful acknowledgment to the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Canada, for providing facilities for the work, and to the Cereal Division, Experimental Farms Service, for seed of certain hybrid lines of wheat. He extends sincere thanks to Dr. S. C. Salmon, Agronomist-in-Charge, Wheat Investigations, United States Department of Agriculture, Washington, D.C., for supplying seed of the Chinese wheats used in the study. He wishes to acknowledge the kind interest of Dr. J. H. Craigie in the study, the helpful advice and criticism of Dr. Thorvaldur Johnson and Dr. Margaret Newton during the course of the work, and the assistance of Dr. R. F. Peterson in planning the field experiments. Finally, the writer wishes to express his sincere thanks to the China Foundation for the Promotion of Education and Culture for the financial aid through which alone it was possible for him to carry out the investigation during these critical years in his homeland.

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VEGETATIVE PROPAGATION OF CONIFERS

XI. EFFECTS OF TYPE OF CUTTING ON THE ROOTING OF NORWAY SPRUCE CUTTINGS¹

By J. L. FARRAR² AND N. H. GRACE³

Abstract

Full length Norway spruce cuttings, with and without a heel of old wood, were collected from the lower part of the tree at semimonthly intervals from July to October and were propagated in several media in outside frames.

Plain cuttings generally rooted better than cuttings with a heel of old wood, as judged by percentage rooted and the number and lengths of root. Heels, however, for summer collections favoured survival and rooting in sand, and root length in sand-peat. In several experiments involving early spring collections propagated in sand, the presence or absence of heels had little effect on the responses of the cuttings.

A late October collection involved six types of plain cuttings taken from the lower part of the tree and propagated in two different sand-peat media. In sedge peat medium there was little difference in the rooting of second order terminal, second order large lateral, second order small lateral, or third order lateral cuttings, the average rooting being 90%. Third order lateral cuttings showed an inferior development of new growth. The percentage of first order terminal cuttings rooted was 67%; these had markedly superior new growth development; when shortened, only 32% of such cuttings rooted. Irregular differences between the types of cutting occurred in the inferior sphagnum peat medium.

An important consideration in the vegetative propagation of conifers is the type of cutting used. Rooting and other responses are influenced by the age of the parent tree, the limb from which the cutting is taken, the part of the branch used to make the cutting, and its vigour (1-4, 6, 7, 9-14). This communication presents information on the reactions of cuttings made from twigs of different branch orders, cuttings with and without two-year old wood, and cuttings including all and part of the current year's growth.

Experimental

The experimental study of the effect of type of cutting on the rooting of Norway spruce involved seven experiments with 8500 cuttings all planted in outdoor frames. The experiments were of factorial design and the results pertaining to chemical treatments, propagation medium, and season of collection have already been presented (5, 8). Those data referred only to cuttings without old wood made from full length twigs 5 to 10 cm. long unless otherwise specified. The experiments designated in this paper were assigned the same number in a previous paper (5). Methods of collecting and planting the cuttings have already been described (8).

¹ Manuscript received November 23, 1941.

Contribution from the Dominion Forest Service, Ottawa, and the Division of Biology and Agriculture, National Research Laboratories. Part of a co-operative project of the Subcommittee on Forest Tree Breeding, Associate Committee on Forestry. N.R.C. No. 1037.

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Experiment 1

Full length cuttings 5 to 10 cm. long from the lower part of the tree with and without a heel of old wood were collected at semimonthly intervals during July and August, 1939, and planted in sand and sand mixed separately with sphagnum and sedge peats (5).

Experiment 2

Semimonthly collections were continued through September and October but were planted in sand and sand mixed with sedge peat only.

Experiment 3

Cuttings were collected from the lower part of the tree and were separated into five types as shown in Fig. 1. These five types of cuttings were all full length and plain, without a heel of old wood. Type 1 refers to first order terminals with an average length of 127 mm.; Type 2, second order terminals, 81 mm.; Type 3, second order large laterals, 93 mm.; Type 4, second order small laterals, 71 mm.; and Type 5, third order laterals, 59 mm. Cuttings of Type 6 are the distal part of Type 1 cuttings and had an average length of 79 mm.

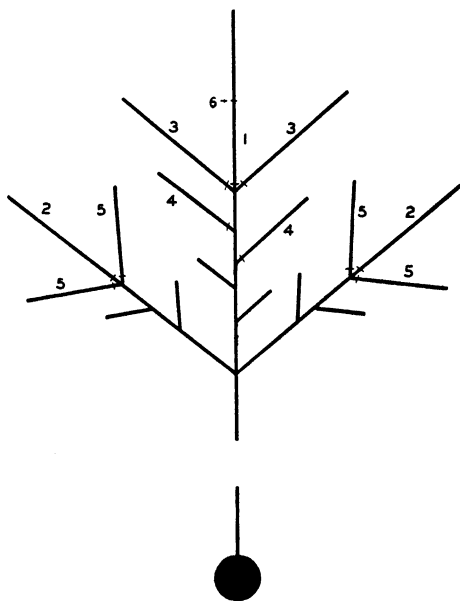


FIG. 1. Diagrammatic representation of position on a lower branch of a Norway spruce tree from which cuttings of current season's growth were taken. Lines across twigs indicate position of cut. Type 6 is the distal part of a Type 1 cutting.

In several experiments cuttings were planted in sand in outdoor frames before, during, and after initiation of new growth in the spring of 1939. Terminal and lateral cuttings were taken from the upper and lower part of

the tree; cuttings were both plain and with a heel of old wood, and some plantings involved full length lateral cuttings with both 1937 and 1938 growth. In 1938, some experiments involved cuttings made only from the lower portion of long twigs.

As indicated in a previous article (5), it has been possible to develop certain additional criteria on the greenhouse propagation in sand of long and short Norway spruce cuttings, plain and with heels. These data were recorded by the senior author at New Haven, Conn., in 1938, and the data subsequently published dealt chiefly with rooting (2, 4).

Cuttings of Experiments 1, 2, and 3 were removed for counts and measurements in September, 1940. Details of the observations made have been given in an earlier article (5). The cuttings planted in sand in the spring of 1939 were removed June, 1940, and counts were made of the number of cuttings surviving, rooted, and with new growth; the number and lengths of root were also determined. The cuttings planted in 1938 were examined in 1939. Statistical treatment of data from the responses of cuttings has been described previously (4, 6-8).

Results

The responses of the different types of cutting of Experiments 1, 2, and 3 are given in Table I. The data for Experiments 1 and 2 are averaged over four collections and four treatments as there were no important interactions between these factors and type of cutting. Data are given for responses in the three main types of media since there were marked interactions between the type of cutting and the propagation medium.

In Experiment 2 plain cuttings, on the average, were superior to those with a heel of old wood with regard to numbers and lengths of roots, and percentage rooted. These differences are illustrated in Fig. 2.

In Experiment 1, heels appeared to favour root length in the peat media, and survival and rooting percentage in sand; otherwise, plain cuttings were equal or superior.

In both Experiments 1 and 2, an almost equally high percentage of each type of cutting rooted in the favourable sedge peat medium.

Sand propagation in the greenhouse at New Haven, Conn., demonstrated that long cuttings had 3.8 roots with a length of 104 mm. per rooted cutting while short cuttings had 3.0 roots and a length of 63 mm. There was no significant difference between plain cuttings and those with a heel in respect to either number or lengths of roots per rooted cutting. Mortality of the long cuttings was substantially greater than that of the short, and was unaffected by heels. This increased mortality is of interest since the long cuttings rooted better than the short (2, 4). Long cuttings developed new growth to a greater extent than short, and the plain were superior to those with heels.

In the experiments done in outdoor frames in sand in the spring of 1939, the presence of heels had little effect, though heels resulted in a slight increase in the mean root length. Of the cuttings with the full length of two years'



2



3

FIG. 2. Norway spruce cuttings propagated in sand. Upper: plain cuttings without a heel of old wood. Lower: cuttings with heel of old wood.

FIG. 3. Norway spruce cuttings propagated in sand-sedge-peat. Upper: shortened first order twigs (Fig. 1, Type 6). Lower: full length first order twigs (Fig. 1, Type 1).



TABLE I

EFFECTS OF TYPE OF CUTTING ON RESPONSES OF NORWAY SPRUCE AND INTERACTION EFFECTS WITH MEDIA

Criteria	Medium	Experiment 1		Experiment 2		Experiment 3					
		Cuttings		Cuttings		Type of cuttings†					
		Plain	With heels	Plain	With heels	1	2	3	4	5	6
Number of cuttings surviving, %	Sand	22	53	92	97						
	Sphagnum peat	78	80			30	62	77	73	47	73
	Sedge peat	98	98	98	98	87	92	93	97	93	53
Number of cuttings rooted, %	Sand	14	20	80	39						
	Sphagnum peat	58	38			13	35	47	47	30	22
	Sedge peat	92	88	95	83	67	88	87	95	90	32
Number of cuttings rooted as percentage of number surviving	Sand	61	37	87	40						
	Sphagnum peat	75	42			44	57	61	64	64	30
	Sedge peat	93	90	97	84	77	96	93	98	96	59
Number of roots per rooted cutting	Sand	3.5	2.9	4.0	2.5						
	Sphagnum peat	3.7	3.7			2.4	4.1	2.8	2.9	3.6	1.8
	Sedge peat	4.7	4.5	4.6	3.0	4.6	4.2	4.5	4.2	3.8	2.7
Length of roots per rooted cutting, mm.	Sand	91	65	107	56						
	Sphagnum peat	149	157			79	175	115	98	137	71
	Sedge peat	220	261	234	133	255	230	277	203	192	89
Mean root lengths, mm.	Sand	26	23	26	22						
	Sphagnum peat	40	43			33	43	41	33	38	38
	Sedge peat	47	58	51	44	56	55	61	48	50	33
Number of cuttings with new growth, %	Sand	4	2	45	42						
	Sphagnum peat	2	2			18	17	15	10	7	23
	Sedge peat	54	46	67	60	87	75	87	72	48	50
Number of cuttings with new growth as a percentage of those surviving	Sand	8	2	49	43						
	Sphagnum peat	3	2			61	27	20	14	14	32
	Sedge peat	54	47	68	62	100	82	93	74	52	94
Number of rooted cuttings with new growth as a percentage of those rooted	Sand	7	3	50	50						
	Sphagnum peat	4	3			50	38	29	18	11	38
	Sedge peat	55	49	69	63	100	81	92	74	52	95
Number of rooted cuttings with new growth as a percentage of those with new growth	Sand	X	X	89	46						
	Sphagnum peat	X	X			36	80	89	83	50	36
	Sedge peat	95	94	98	86	77	96	92	98	97	60
Number of shoots per cutting with new shoots	Sand	X	X	1.2	1.1						
	Sphagnum peat	X	X			1.3	1.3	1.0	1.0	1.0	1.0
	Sedge peat	1.4	1.4	1.4	1.2	3.1	1.4	1.6	1.2	1.1	1.7
Mean length of longest shoot on each cutting with new shoots, mm.	Sand	X	X	19	17						
	Sphagnum peat	X	X			26	16	26	15	11	17
	Sedge peat	21	21	28	23	41	35	41	29	34	33

† See Fig. 1.

X = Meagre data.

growth, 91% survived the test period, but only 30% were rooted; survival of full length plain cuttings of one year's growth was 89%, with 41% rooting.

It is apparent from the data of Experiment 3 that there were only small differences between the rooting responses of Types 2, 3, 4, and 5 when propagated in a sedge peat medium, though third order lateral cuttings (Type 5) were rather poor in respect to development of new growth. Somewhat greater differences occurred among these types when planted in sphagnum peat. The first order terminal cuttings, Type 1, were definitely below Types 2, 3, 4, and 5 in respect to percentage rooted, were quite equal to them in respect to numbers and lengths of roots, and superior in respect to new growth. When shortened to a length approximately equal to Types 2, 3, and 4, such first order terminal cuttings (Type 6) were much inferior to the other types, especially when planted in the superior sedge peat medium. It might be noted that practically all surviving cuttings of Types 1 and 6 bore new growth. However, for all classes of cuttings, those surviving rooted equally well whether they bore new growth or not. Furthermore, comparison of percentage rooted and percentage with new growth shows that a type of cutting favouring one condition does not necessarily favour the other. The effects of shortening first order terminal cuttings are illustrated in Fig. 3, which shows the living cuttings from representative groups of 10 cuttings.

Spring plantings in sand confirmed earlier results on the effect of position of the cutting on the tree and on the branch (6, 13). Cuttings taken from the lower part of the tree rooted better than those from the upper region and lateral cuttings were consistently superior to the terminals.

Some cuttings made from the lower part of the twig, that is, without a terminal bud, and planted in a mixture of sand and sphagnum peat in 1938 did root, though the percentage was small. In this connection it may be noted that cuttings in which the new growth all died (8) were not prevented from rooting. This indicates that the terminal bud is not essential to the rooting of cuttings.

Discussion

The results suggest that it is important that cuttings be made the full length of the current year's growth. Though short cuttings might be superior to long, the latter were not improved by shortening them. There was no advantage in making cuttings with a heel.

The position of the cutting on the branch had little effect if the cuttings were made from twigs about 6 to 9 cm. long and planted in the sedge peat medium. Long terminal cuttings also rooted in fair percentages in the same medium. Since these larger cuttings show good development of roots and new growth, their propagation may be of value in overcoming the plagiotropic growth habit evidenced by spruce cuttings.

The greater development of new growth in sedge peat medium suggests that substances absorbed by the cutting affect bud development. The reduction in new growth when cuttings are shortened may possibly be related to the nutrients available in the twig.

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HYBRIDIZATION OF *TRITICUM* AND *AGROPYRON*

VII. NEW FERTILE AMPHIDIPOIDS¹

By F. H. PETO² AND G. A. YOUNG³

Abstract

Fertility was induced by means of colchicine treatments in sterile intergeneric hybrids of the following wheat varieties crossed with *Agropyron glaucum*:

T. vulgare var. Mosida, Ruby, Milturum, and Kharkov

T. durum var. Mindum and Black Persian

T. turgidum

T. dicoccum var. Vernal

T. pyramidale

Three methods of applying colchicine were compared and the "capsule" method was found to be the most economical and effective.

Introduction

A fertile amphidiploid of *Triticum vulgare* var. Kharkov \times *Agropyron glaucum* was produced in 1937 by heat treatments (3). Colchicine treatments were used with slightly better results in 1938 and 1939 when fertile amphidiploids of Vernal emmer \times *A. glaucum* were produced (4). Cytological studies at that time revealed that the chromosome number was approximately doubled and that a high proportion of the chromosomes formed bivalents at the reduction division. The method of applying the colchicine was similar to that used by Myers (2). Dry seed was placed in Petri dishes on blotting paper moistened with 0.1, 0.2, or 0.4 % aqueous solution of colchicine for 24 hr.

In the experiments reported below, Myers' treatment was again used and compared with two other methods. One of these was suggested by Dorsey (1) and consisted of germinating the seeds on moist blotting paper in a germinating chamber at 18° C. At various stages from emergence of the epicotyl until it reached 20 mm. in length, the seedlings were immersed in a 0.2% solution of colchicine for various durations from 5 to 50 min. In some cases penetration

¹ Manuscript received December 11, 1941.

Contribution from the Division of Biology and Agriculture, National Research Laboratories, Ottawa, Canada. This contribution forms part of a co-operative investigation on the hybridization of *Triticum* and *Agropyron* undertaken by the Dominion Experimental Farm and the National Research Council of Canada. N.R.C. No. 1040.

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of the solution was facilitated by the use of a partial vacuum which permitted the use of the shorter treatment periods. This method was frequently used following Myers' seed treatment when the resulting seedlings failed to show any effect.

A capsule method devised for use on dicotyledonous plants was also used on seedlings just prior to tillering. This treatment was used only on plants on which neither the seed treatment nor immersion method appeared to have been effective. Gelatine capsules, commonly used for pharmaceutical preparations, were coated with collodion to prevent dissolution. These were filled with an agar gel solution containing 0.1 or 0.4% of colchicine. The stems of the seedlings were cut off about two centimetres above the ground level and the capsules containing the gel were slipped over this short stem. In this way the colchicine was absorbed into the stem and presumably affected the primordia of the new tillers being formed at nodes just below the ground level.

Observations

A total of 273 F_1 hybrid seeds of various *Triticum-A. glaucum* crosses produced at the Division of Forage Plants, Central Experimental Farm, Ottawa, Canada, were treated either as seeds or seedlings. These hybrids are listed in Table I in which is also recorded the number of fertile plants resulting from all the treatments. Twenty-eight fertile plants were produced, which is 10.3% of the seeds or seedlings treated. These results are very much better than those obtained the previous year (4) by Myers' seed

TABLE I

F_1 HYBRID MATERIAL USED FOR COLCHICINE TREATMENTS AND THE NUMBER OF FERTILE PLANTS PRODUCED

Hybrid	Number of seeds	Chromosome number	Number of fertile plants
Turkey red × <i>A. glaucum</i> (1087)	5	42	0
Yaroslav × <i>A. glaucum</i> (1087)	7	35	0
Mosida × <i>A. glaucum</i> (1087)	20	42	1
Mosida × <i>A. glaucum</i> (A38)	20	42	0
Kharkov × <i>A. glaucum</i> (A38)	5	42	1
Ruby × <i>A. glaucum</i> (1087)	6	42	2
Milturum × <i>A. glaucum</i> (1087)	24	42	1
<i>T. pyramidale</i> × <i>A. glaucum</i> (1087)	11	35	1
<i>T. turgidum</i> 49 × <i>A. glaucum</i> (1087)	12	35	3
<i>T. turgidum</i> 49 × <i>A. glaucum</i> (A38)	11	35	4
Vernal emmer × <i>A. glaucum</i> (1087)	2	35	1
Vernal emmer × <i>A. glaucum</i> (A38)	17	35	4
Mindum × <i>A. glaucum</i> (1087)	57	35	3
Mindum × <i>A. glaucum</i> (A38)	61	35	6
Black Persian × <i>A. glaucum</i> (A38)	8	35	1
Black Persian × <i>A. intermedium</i> (A39)	7	35	0
Totals	273		28

NOTE: Average success = 10.3%.

TABLE II
FERTILITY OF AMPHIDIPLOIDS PRODUCED IN THE GREENHOUSE

Plant No.	Hybrid	Total number spikes	Effective* treatment	Number fertile spikes	Order of emergence of fertile spikes	Dehiscence	Number seeds	Total number spikelets	Percentage fertility
22-4	Milturum × <i>A. glaucum</i> (1087)	8	II	3	4, 5, 6	Fair	13	108	12.0
23-1	<i>T. pyramidalis</i> × <i>A. glaucum</i> (1087)	5	IV	2	1, 3	Good	17	85	20.0
25-2	<i>T. turgidum</i> 49 × <i>A. glaucum</i> (A38)	13	IV	2	1, 4	Good	22	68	32.4
25-3	<i>T. turgidum</i> 49 × <i>A. glaucum</i> (A38)	4	IV	2	2, 4	Good	7	56	12.5
26-2	Vernal emmer × <i>A. glaucum</i> (A38)	7	IV	1	3	Good	1	32	3.1
26-6	Vernal emmer × <i>A. glaucum</i> (A38)	10	IV	3	1, 4, 5	Good	9	104	8.7
39-1	<i>T. turgidum</i> 49 × <i>A. glaucum</i> (A38)	7	IV	1	2	Good	1	30	3.3
40-1	Mindum × <i>A. glaucum</i> (1087)	3	III	1	1	Good	9	24	37.5
42-1	Vernal emmer × <i>A. glaucum</i> (A38)	6	III	1	3	Good	3	34	8.8
48-1	<i>T. turgidum</i> 49 × <i>A. glaucum</i> (1087)	8	III	1	2	Good	18	36	50.0
51-4	Mindum × <i>A. glaucum</i> (A38)	6	III	1	1	Poor	16	36	44.4
51-6	Mindum × <i>A. glaucum</i> (A38)	14	III	5	1, 5, 6, 7, 14	Fair	26	130	20.0
51-7	Mindum × <i>A. glaucum</i> (A38)	9	III	1	2	Poor	13	32	40.6

* Effective treatments: I = Myers; II = Myers and immersion; III = immersion; IV = capsule.

treatment method which resulted in only one fertile plant from 237 treated seeds. When the percentage of success from treatments of pentaploid ($2n = 35$) and hexaploid ($2n = 42$) hybrids was calculated separately, it was found that fertility occurred in 11.9% of the pentaploids and only 6.2% of the hexaploids.

The plants that exhibited fertility in the greenhouse subsequent to treatment are listed in Table II. It will be seen that the immersion method (III) and the capsule method (IV) gave the best results and that all but one of the amphidiploids were pentaploids.

Records were kept on the order of emergence of the tillers bearing partially fertile spikes. It might be assumed that the first tiller appearing after treatment would most likely exhibit fertility, particularly where the capsule method (IV) was used. Actually any of the first six tillers produced in the greenhouse were likely to be partially fertile. In fact, fertility may not appear until the second crop as is shown by the occurrence of fertile spikes in plants that had been entirely sterile in the greenhouse and were then transplanted to the field.

The anthers on the fertile sectors of the plant were large, plump, and dehiscent normally, while the anthers on the sterile portions were small and thin and never shed any pollen. Consequently the fertile sectors of the spike could be readily detected at the time of flowering. In one spike, only a single anther dehiscent which shows how small the amphidiploid sector can be and still result in fertility. At the other extreme, whole spikes appeared to be entirely amphidiploid since practically all of the anthers dehiscent normally. This situation is reflected in the percentage fertility recorded in Table II where the figures vary from 3.1 to 50%.

After harvesting the seed from all the plants exhibiting fertility in the greenhouse, these plants plus 32 others that failed to show any signs of fertility in the greenhouse (two from each hybrid) were transferred to the field. At harvest all the plants were again checked for fertility.

Twelve of the 13 plants that were fertile in the greenhouse were sterile in the field. This indicates either an elimination of the amphidiploid tissue or a tendency for tillers to arise from undoubled sectors of the plant. The lone plant that was fertile in the field was No. 48-1, *T. turgidum* \times *A. glaucum*. Of the 32 treated plants that failed to show signs of fertility in the greenhouse, 15 (or 47%) were partially fertile in the field. This shows that an unexpectedly high proportion of amphidiploid tissue must have existed in the plants that failed to produce seed in the greenhouse. If this delayed expression of fertility had been suspected, then all the treated plants would have been transplanted and the average success of the treatments would have been much higher than 10.3%.

There are at least three obvious factors influencing the degree of success of the treatments. These may be stated as follows: (1) the proportion of amphidiploid tissue induced by treatment; (2) the location of the amphi-

TABLE III
FERTILITY OF AMPHIDIPOIDS PRODUCED IN THE FIELD

Plant No.	Hybrid	Effective treatment	Number of fertile heads	Number of seeds	Wt. per 1000 kernels, gm.
19-2	Mosida × <i>A. glaucum</i> (A38)	I or II	2	4	13.0
20-2	Kharkov × <i>A. glaucum</i> (A38)	I	4	9	16.4
21-1	Ruby × <i>A. glaucum</i> (1087)	IV	4	59	13.5
21-2	Ruby × <i>A. glaucum</i> (1087)	IV	2	18	15.7
25-4	<i>T. turgidum</i> 49 × <i>A. glaucum</i> (A38)	I or II	1	1	10.0
25-5	<i>T. turgidum</i> 49 × <i>A. glaucum</i> (A38)	I or II	2	2	13.3
26-7	Vernal emmer × <i>A. glaucum</i> (A38)	I	1	1	15.0
29-2	Black Persian × <i>A. glaucum</i> (A38)	I or II	9	167	15.8
31-5	Mindum × <i>A. glaucum</i> (A38)	III	1	2	15.0
32-1	Mindum × <i>A. glaucum</i> (A38)	III	3	3	13.3
43-1	Mindum × <i>A. glaucum</i> (1087)	III	8	8	18.5
43-2	Mindum × <i>A. glaucum</i> (1087)	III	1	1	20.0
48-2	<i>T. turgidum</i> 49 × <i>A. glaucum</i> (1087)	III	2	2	16.0
49-1	Vernal emmer × <i>A. glaucum</i> (1087)	III	1	1	19.0
51-8	Mindum × <i>A. glaucum</i> (A38)	III	1	1	11.2

TABLE IV
RELATIVE EFFECTIVENESS OF TREATMENTS APPLIED BY DIFFERENT METHODS*

Treatments	Number plants surviving treatment	Number fertile plants	Percentage
I (Myers)	55	2	3.6
II (Myers plus immersion)	47	1	2.1
III (Immersion)	99	13	13.1
IV (Capsule)	37	8	21.6

* Four fertile plants from either treatment I, II, or both combined not recorded above.

diploid tissue in the primordia of the spike; (3) the competition between normal and amphidiploid tissue during development. The interaction of these three factors probably accounts for the inconsistencies noted above.

A comparison of the effectiveness of the four treatments is given in Table IV. In assessing the relative merits of these treatments, it should be kept in mind that the cumulative effect of a second treatment on the same plant would bias to some extent the conclusions in favour of the last treatment which was credited with the results in those plants where no favourable response was apparent from the first treatment. This situation could not be avoided since it was essential to use this valuable and limited hybrid material as economically as possible. In spite of these limitations, the following conclu-

sions appear to be justified. The capsule method of application of colchicine was the most effective, 21.2% of the plants that survived treatment showing fertility. It also had the advantage of being least wasteful of valuable hybrid seed since the plants were well established before application of the colchicine. The cutting back of the primary shoot just prior to normal tillering actually stimulates tiller development and the colchicine is absorbed into the region of the stem where the primordia of the new tillers are being formed. The immersion method is very effective but the roots of the young seedlings are needlessly damaged. The chief criticism of the seed treatment method (Myers) is that it is wasteful of seed since the most effective dosage frequently kills up to 50% of the seedlings.

The weights of the amphidiploid seed are shown in Table III. The *A. glaucum* seed usually weighs between 5 and 6 gm. per 1000, the amphidiploid hybrid seed varies from 10 to 20, while the weights of seed of the wheat parents vary from about 32 to 37 gm. per 1000.

The agronomic possibilities of these new amphidiploids have not yet been determined but they will be multiplied and distributed for test as soon as possible. The Vernal-*A. glaucum* amphidiploid was also produced from treatments applied in 1938 and subsequently has been multiplied and tested to a limited extent at Ottawa, Saskatoon, Edmonton, and Vancouver. Sufficient seed is now available for extensive yield trials for hay and pasture at several points in Canada. These trials will be organized and supervised by the Division of Forage Plants, Central Experimental Farm, Ottawa. The observations on the preliminary trials show that this amphidiploid is strictly perennial and winter hardy at all the above locations. It is very leafy, shows some signs of being drought resistant, and is surprisingly uniform in regard to most morphological characters. The fertility is very high and good yields of seed have been obtained. Its main fault appears to be the shattering rachis and persistent glumes which were inherited from the emmer parent. These characters prevent the seed from being readily separated from the glumes with an ordinary threshing machine. However, this is the only fertile hybrid yet obtained that would cause much difficulty in this regard and it should be possible to breed out this undesirable character through hybridization with one of the more readily threshable amphidiploids.

In general, the results obtained to date indicate that fertility can be readily induced in sterile F_1 hybrids of any of the wheat varieties crossed with *A. glaucum* through doubling the chromosome number by means of colchicine treatments. Prior to the development of this new method of producing fertile amphidiploids, improvement was limited to selection and hybridization within and between closely related species. Now the possibility of combining characters from related genera has become a reality and the production of new and strikingly different forage crops is in the process of being realized.

Acknowledgments

The authors are indebted to Dr. T. M. Stevenson, Dr. J. M. Armstrong, and Mr. A. McLennan of the Division of Forage Plants, Central Experimental Farm, Ottawa, Canada, for supplying hybrid seed and for generous co-operation at all times.

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ULTRACENTRIFUGE AND DIFFUSION STUDIES ON GLUTEN¹A. G. MCCALLA² AND NILS GRALÉN³

Abstract

The molecular characteristics of gluten in sodium salicylate solutions were studied by means of sedimentation velocity, sedimentation equilibrium, and diffusion measurements. The proportion of total gluten protein molecularly dispersed increased with increase in concentration of sodium salicylate up to 12%, but the dispersed portions had essentially the same sedimentation constant (2.5 ± 0.15) regardless of the concentration of the dispersing medium.

The most soluble 25 per cent of the gluten was all molecularly dispersed, but was definitely inhomogeneous. The weight-average molecular weight of this fraction was 44,000, but there is reason to believe the minimum weight may be about 35,000. None of the other fractions was entirely molecularly dispersed, the proportion decreasing with decreasing solubility of the fractions. Aggregates of many sizes existed in all of these fractions, but only the most insoluble contained aggregates large enough to cause opacity. Sedimentation constants of the molecularly dispersed portions increased slightly with decreasing solubility, while diffusion constants decreased markedly. None of the fractions yielded normal curves (diffusion diagrams) but the more soluble the fraction, the more nearly normal the curve. The inhomogeneity responsible for the varying rates of diffusion was due partly to differences in proportion and properties of the molecularly dispersed gluten and partly to aggregates.

All properties showed progressive changes both within and between the arbitrarily produced fractions. These results, therefore, support the hypothesis that gluten is a protein system showing progressive and regular changes in properties with change in solubility.

Introduction

In a recent note (17) attention was called to a few of the results obtained in a study of gluten protein using ultracentrifuge and diffusion methods. It was pointed out that Osborne's conclusion (19) that gluten is made up of two proteins, glutenin and gliadin, has been rendered doubtful by more recent work, but no attempt was made to cover this later literature. The more important results may be briefly summarized as follows:

From 1929 to 1931, there appeared a number of papers in which results that were apparently irreconcilable with the earlier conclusions were published (4, 6, 11, 12, 25). Various explanations were offered to account for the disagreement in results, the most plausible one being suggested by the results of Haugaard and Johnson (11) and enlarged upon as part of a general discussion by Sørensen (25). According to this hypothesis, gliadin is a protein system which can be reversibly fractionated, and is not an individual chemical compound.

That the original classification of Osborne is inadequate to explain the make-up of gluten was further shown by Sandstedt and Blish (23), who advanced the hypothesis that gluten was made up of three main dissociable component

¹ Manuscript received November 3, 1941.

Contribution from the Institute of Physical Chemistry, University of Uppsala, Sweden, and the Department of Field Crops, University of Alberta, Edmonton, Alta., with financial assistance from the National Research Council of Canada, Rockefeller Institute, Searle Grain Company, Winnipeg, Man., and Wallenberg Foundation. Published as Paper No. 192 of the Associate Committee on Grain Research.

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systems, each in turn consisting of a group of lesser dissociable complexes. This system, under their conditions, was not reversible. McCalla and Rose (18), using gluten preparations in sodium salicylate solutions, and fractional precipitation methods, were able to get complete reversibility in the fractionation. It was concluded that gluten was a single protein complex. This conclusion was criticized by Blish (3) who reported that results obtained by fractional solubility studies not only failed to substantiate those obtained by McCalla and Rose, but definitely contradicted the evidence afforded by the fractional precipitation data.

At this time the first results obtained with wheat proteins using ultracentrifuge and diffusion methods were reported (13, 14, 16). These agreed with the hypothesis that gliadin was not a chemical individual, and the diffusion results definitely supported the conclusions of McCalla and Rose as far as they applied to the gliadin portion of the gluten. Results of solubility and swelling studies carried out by Kuhlmann (15) led him to conclude that gluten, considered as a natural high polymer, represents a complex of proteins forming micelles of various lengths. The more insoluble portions of the gliadin are made up of longer micelles than the more soluble, but glutenin micelles are still longer than these. The β -gliadin (less soluble) approaches glutenin in its properties. It is significant that the author shows a considerable number of micelle lengths in diagrammatically representing his ideas regarding the nature of the gluten components.

The osmotic pressure of gliadin solutions was studied by Burk (5). The results showed that variation in precipitation temperature resulted in variation in the osmotic pressure of the prepared gliadin, and Burk concluded that gliadin was inhomogeneous. He also found that the number-average molecular weight* of well purified gliadin was 41,000 in alcoholic, and 44,000 in urea solutions. In a buffered solution of 75% glycerol at 30° C., the number-average molecular weight was 67,000; in buffered urethane solution at 0° C., 75,000; and in salt-free urethane at 25°, 42,000. He ascribed the higher results to the presence of aggregates which he says were presumably formed from coagulated gliadin molecules present in the preparations.

While all of these results agreed in showing that gliadin is not a homogeneous substance, they did not afford direct evidence as to the nature of the whole gluten complex, nor were they of immediate value in appraising the validity of Blish's criticisms (3). Further work with sodium salicylate dispersions of whole gluten utilizing solubility methods instead of precipitation in obtaining fractions led Spencer and McCalla (26) to conclude that Blish's failure to get concordant results with the two methods was probably due to the ease with which this protein material is denatured. The resulting loss of solubility interferes greatly in the separation of the fractions. By modifying

* The number-average molecular weight is the average molecular weight obtained by any method that in effect involves the counting of molecules (e.g., osmotic pressure). The weight-average molecular weight, in contrast, is the average molecular weight obtained by any method that involves the weights of the molecules (e.g., ultracentrifuge). For homogeneous material the two averages are the same, but for heterogeneous material the weight-average is greater, the magnitude of the difference increasing in general with increased heterogeneity (27, p. 343).

the method of making extractions of the gluten, it was possible to get results that agreed very well with those obtained earlier in precipitation studies.

The results obtained by Krejci and Svedberg using the ultracentrifuge (13, 14) and by Lamm and Polson (16) using diffusion suggested that these methods should yield more definite information regarding the fundamental physical make-up of gluten than had hitherto been obtained. The results of such studies carried out at Uppsala are reported in this paper.

Material and Methods

Two flours were used as the source of the gluten for most of the experiments. The first was a commercial patent flour milled in Sweden. This flour contained 10% protein and yielded gluten typical of soft wheat, i.e., it was soft and very extensible, but of good quality in all other respects. The second flour used was experimentally milled from Marquis wheat grown at the University of Alberta, Edmonton, Alta., in 1939. This flour contained 16% protein and yielded gluten that was of excellent quality in all respects, being firm, elastic, and extensible. In this paper, the first flour is referred to simply as "soft", the second as "Marquis".

The methods of preparation, fractionation, and analysis follow closely those described in detail in earlier papers (18, 26). For most of the experiments, dispersion was carried out in 8% sodium salicylate solution. Most gluten preparations were washed from the flour using the method and phosphate buffer of pH 6.8 described by Dill and Alsberg (8) although 0.25% sodium salicylate of pH 6.5 was substituted in some of the later preparations because this resulted in the removal of a larger proportion of the non-gluten proteins (18). After the first few preparations had been studied, the method of dispersion was modified to involve as little mechanical manipulation as possible, since it was found that the gluten was extremely sensitive to such action and was decidedly denatured by treatment much less severe than that used in earlier experiments (18, 26). Such denaturation was not indicated by solubility changes, but rather by changes in the shape of the sedimentation diagrams. Illustrations of this effect are presented in the results.

Sedimentation velocity determinations were carried out using the high-speed, oil-turbine ultracentrifuge at 65,000 r.p.m. (centrifugal force about 300,000 times gravity). The temperature was not precisely controlled, but varied only between 24° and 28° C. for all but a few experiments. All measurements were made using the scale method developed by Lamm (27, p. 254) and, wherever possible, concentrations were calculated from the sedimentation diagrams (27, p. 295).

Sedimentation equilibrium determinations using the low-speed ultracentrifuge were carried out at various speeds. The temperature was kept at 20° C., and all measurements were made by the scale method.

Diffusion determinations were carried out using the steel cell and scale method of measurement described by Lamm (16 and 27, p. 254). The cal-

culations of diffusion constants were made by the statistical method described by Lamm and Polson (16) which gives a weight-average for the diffusion constants of a polydisperse system. In addition the Formula 4 of Lamm and Polson (16) was used to give the variation of the diffusion constant along the experimental curve. All but two experiments were made at 20° C., and these two were corrected for the higher temperature and are reported as for 20° C.

The reasons for selecting sodium salicylate as the dispersing reagent for most of the experiments are much the same as those already discussed fully elsewhere (7, 18, 22). This reagent is the only one of those that have been used extensively for dispersing gluten that is even reasonably satisfactory for use in the ultracentrifuge. Experiments with acetic acid, and with acetic acid-alcohol-sodium-acetate solutions are described in this paper. Urea must be used in such high concentrations that satisfactory results are difficult to obtain from ultracentrifuge studies. It is also necessary to add small quantities of an electrolyte and this reduces the dispersing power of the urea.

Results

PRELIMINARY

The first series of experiments was carried out using the high-speed ultracentrifuge. A crude preparation of gluten in 8% sodium salicylate was centrifuged at 3000 r.p.m. for 20 min. to remove starch, and then used directly in the ultracentrifuge. The results obtained showed that the method could be expected to give good results. No other salt was needed as the salicylate itself removed the electrostatic effects. The results are given in Table I, run No. 2. As prepared, the dispersion was opaque, but it clarified rapidly as the ultracentrifuge speed increased and was clear before 20,000 r.p.m. was

TABLE I

PRELIMINARY CENTRIFUGE RUNS, SOFT FLOUR GLUTEN. ALL DISPERSIONS IN 8% SODIUM SALICYLATE

Centrifuge run No.	Material	Protein** concentration, %	Temperature, °C.	s_{20}^{\dagger}	Protein molecularly dispersed, %
2	Crude gluten	0.68	33	2.69	73
12	Crude gluten	2.00	28	2.59	68
13	Crude gluten	1.00	28	2.60	68
22	Gluten from ether extracted flour	1.06	28	2.47	68
23	Gluten from ether extracted flour	1.12	28	2.54	64
49*	Gluten from ether extracted flour	1.20	26	2.47	64

* Same preparation as No. 22, but stored in refrigerator at 4° C. for three and one-half months.

** Unless otherwise stated, concentration of protein in dispersions was determined by the Kjeldahl method using: protein = Kjeldahl nitrogen \times 5.7, this being the accepted conversion factor for wheat, flour, etc.

† Sedimentation constants are reported in units of 10^{-13} cm./sec. dyne.

reached. During the first 20 min. after reaching full speed (65,000 r.p.m.) there was evidence that small amounts of heavier components were sedimenting faster than the main one. There was also evidence that fat was rising to the top of the column, and this was substantiated by the shape of the sedimentation diagram.

Although the runs listed in Table I were not made consecutively, it is convenient to consider them together as they represent "preliminary" information required at various stages of the work.

Another dispersion was made up in 8% sodium salicylate and two aliquots prepared, one containing 2% protein and the other 1%. The results are also given in Table I, Runs 12 and 13. These results agree excellently and show that concentration did not affect either the sedimentation constant (s_{20}) or the proportion of the protein that was molecularly dispersed*. During subsequent experiments dispersions of lower concentration were frequently used. There was no evidence that the s_{20} values were affected. The amount of protein so dispersed appeared to be lower than in run No. 2, and this was to be expected from the relation between temperature and aggregation found by other workers (11, 23) but there is some question as to the significance of the difference. Whether the difference between the s_{20} values has any significance is doubtful.

The effect of the fat on the sedimentation diagrams made concentration calculations difficult as the base-lines were not well defined. Previous work had shown that ether extraction of flour before the gluten was washed had no effect on gluten quality (24). This conclusion is substantiated by the results for Runs 22 and 23 which were made on two entirely distinct preparations. Except that the flour had been ether extracted, the preparations were treated in the same way as that used in run No. 13. The variation of 4% in the molecularly dispersed protein cannot be considered important, as it is doubtful if the concentrations can be determined to closer than 5%. This is particularly true when the solvent is a salt solution as concentrated as 8% sodium salicylate, which by its own sedimentation causes uncertainty as to the position of the base-lines of the curves.

The preparation used in run No. 22 was stored for 3.5 months in a refrigerator at approximately 4° C. During this time, there was some evaporation and the dispersion became quite clear owing to settling of the less completely dispersed protein. The preparation was well shaken and dialysed against 8% sodium salicylate to correct for the increased concentration resulting from evaporation. The results obtained with run No. 49 indicate that there had been no material change in the protein during storage.

* The term "molecularly dispersed" is applied throughout this paper to the portion of the gluten protein that formed the main component in the sedimentation diagram. It will be shown later that the remainder of the protein existed in various degrees of aggregation, and this term distinguishes between the aggregated and non-aggregated portions. The values given are usually the mean from at least three different diagrams obtained at different times after full speed was reached. The concentration of this molecularly dispersed protein was determined from the area under the curve and the specific refractive increment. The latter value was determined during the course of the present investigation and found to agree very well with the values for other proteins. (Refractive index = 188×10^{-4}).

The sedimentation diagrams obtained after 120 min. at full speed during Runs 13 and 22 are shown in Fig. 1. These illustrate the improvement in the diagram resulting from ether extraction of the flour. Neither diagram is as satisfactory as those obtained for many proteins in dilute salt solutions (27), but the sedimentation constant can be calculated just as accurately, and the protein concentration reasonably well.

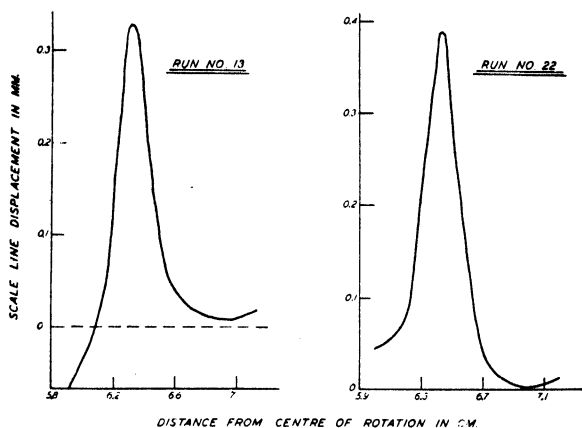


FIG 1. Sedimentation diagrams from non-extracted (run No. 13) and ether extracted (run No. 22) soft flour.

Two other results are worth noting at this point and should be borne in mind during the following discussion.

First, the small amounts of heavier, faster-moving components already mentioned were very indefinite as to sedimentation rate, size, and general occurrence. Results were not reproducible, and often it appeared that sedimentation was negative. This suggested that the apparent components

TABLE II

EFFECT OF TIME OF CENTRIFUGING ON PERCENTAGE PROTEIN CONCENTRATION, CALCULATED FROM SEDIMENTATION DIAGRAMS

Run No.	Concentration of protein, %	Dispersing reagent	Time after reaching full speed, min.			
			40	80	120	180
12	2.00	8% salicylate	1.48	1.32	1.28	—
13	1.00	8% salicylate	0.81	0.65	0.70	—
34	1.14	8% salicylate	—	0.76	0.67	0.66
35	1.19	8% salicylate	—	0.62	0.55	0.53
63	0.83	8% salicylate	—	0.68	0.55	0.44
46*	0.99	12% salicylate	1.00	1.00	0.92	0.77
61*	0.52	12% salicylate	—	0.51	0.44	0.37
26	1.18	Acetic acid	—	0.94	0.88	0.69

* Fractions.

might not be real, rather, that the scale line displacement was due to convection. There did not appear to be any advantage to be gained by making detailed calculations for each run, so after the first few runs they were omitted, although exposures permitting such calculations to be made were always taken.

Second, there was usually a decrease in the concentration values obtained from the successive sedimentation diagrams. This is illustrated by the results for a few runs as given in Table II. Frequently the early diagrams were definitely asymmetrically expanded towards the right-hand side while much of the asymmetry disappeared in later diagrams but did not give rise to any definite components. Such disappearance causes a decrease in the total amount of protein molecularly dispersed, and this result was usually obtained experimentally.

PARTIAL SPECIFIC VOLUME OF GLUTEN

In the calculation of molecular weights using sedimentation and diffusion data it is necessary to use the partial specific volume of the protein. This value is relatively constant at 0.745 to 0.750 for many proteins in aqueous solutions (27). The results of the determinations carried out at Uppsala are presented in Table III. Unextracted Marquis flour was used and all measurements made at 20° C. The values in the first part of the table were

TABLE III

PARTIAL SPECIFIC VOLUME OF GLUTEN FROM EXTRACTED MARQUIS FLOUR AT 20° C.

Preparation No.	Material	Salicylate concentration, %	Protein concentration, %	V
1	Whole gluten, clarified*	8	2.391	0.700
2a	Whole gluten, partially clarified**	8	2.535	0.699
b	Whole gluten, partially clarified	8	1.266	0.698
c	Whole gluten, partially clarified	8	0.633	0.696
3	As for 2a	12	2.530	0.690
4	Fraction BIII, second fractionation experiment	12	2.160	0.690
5	Whole gluten, partially clarified**	8	2.932	0.701
Determinations by Professor C. Drucker				
5a	Preparation 5 above	8	2.932	0.7016
b	Preparation 5 above (diluted)	8	2.423	0.7018
c	Preparation 5 above (diluted)	8	1.925	0.7017
d	Preparation 5 above (diluted)	8	1.447	0.7013
e	Preparation 5 above (diluted)	8	1.074	0.7009
f	Preparation 5 above (diluted)	8	0.738	0.7003
g	Preparation 5 above (diluted)	8	0.510	0.6999
h	Preparation 5 above (diluted)	8	0.280	0.6994

* Clarified in low-speed ultracentrifuge at 18,000 r.p.m. for 24 hr.

** Clarified at 3,000 r.p.m. for three hours.

determined by the senior author while those in the second part were determined by Professor C. Drucker, using a pycnometer of approximately 40 cc. volume and an interpolation formula (9).

These values are very low as compared with those for most proteins, and even lower than that for gliadin reported by Krejci and Svedberg (13). Since the values obtained using 12% salicylate as the solvent are less than those using 8%, it seemed probable that the solvent had a definite effect on partial specific volume.

This was further investigated (at Edmonton) using gluten from another sample of Marquis flour. These determinations were carried out at 25° C. The first preparation was dispersed in 8% sodium salicylate and aliquots dialysed against 2, 4, 6, 8, and 12% salicylate solutions. The second was dispersed in 0.1 *N* acetic acid, one aliquot dialysed against this solution, and a second made up to 60% alcohol and dialysed against a solution of 0.025 *N* acetic acid and 0.05 *N* sodium acetate in 60% ethyl alcohol. All determinations were carried out using pycnometers of approximately 40 cc. volume. The results are given in Table IV. The values for 12 and 8% salicylate agree well with those in Table III although there is less difference between the

TABLE IV
PARTIAL SPECIFIC VOLUME OF GLUTEN FROM EXTRACTED MARQUIS FLOUR AT 25° C.

Preparation No.	Solvent	Solvent concentration	Protein concentration, %	<i>V</i>
6a	Sodium salicylate	12%	1.990	0.699
b	Sodium salicylate	8%	3.392	0.703
c	Sodium salicylate	6%	1.409	0.711
d	Sodium salicylate	4%	2.135	0.714
e	Sodium salicylate	2%	1.289	0.717
7a	Acetic acid	0.1 <i>N</i>	1.929	0.735
	(Acetic acid)	0.025 <i>N</i>		
b	(Ethyl alcohol)	60%	1.284	0.728
	(Sodium acetate)	0.05 <i>N</i>		

values for these two concentrations. It seems likely that the value for 8% is slightly low, since the other four values for salicylate yield a straight line when plotted, while the result for 8% falls about 0.003 below this line. This may be the result of the rather high concentration of protein since Professor Drucker found a maximum value of *V* at a protein concentration of 2.3% for the conditions under which his determinations were made. There is no question but that salicylate concentration affects the partial specific volume, but even the value for 2% salicylate is far below that obtained for most proteins.

The results for the two other solvents, although higher, indicate that the partial specific volume of this protein is less than that for many other proteins regardless of the dispersing reagent.

For purposes of calculating molecular weights the value $V = 0.700$ is used for 8% salicylate and $V = 0.690$ for 12% salicylate.

FIRST FRACTIONATION EXPERIMENTS

The first fractionation experiments were carried out using gluten from unextracted soft wheat flour. In the first, a gluten dispersion in 8% sodium salicylate was dialysed against 1% salicylate with frequent changes of solution. There was a rapid precipitation of protein, and this precipitate was redispersed in 8% salicylate. The material remaining in solution was redialysed against 8% salicylate, and a centrifuge run made with each of the two "fractions". The results are given in Table V, Runs 3 and 4. These runs, carried out at 34° to 35° C., yielded results that were much the same as for the whole gluten except that the proportion of the larger fraction (Run 4), which was molecularly dispersed, was definitely less. This was expected, since the most soluble portion of the dispersion had been removed.

TABLE V

SEDIMENTATION CONSTANTS OF GLUTEN FRACTIONS FROM SOFT WHEAT FLOUR, ALL RUNS MADE IN 8% SODIUM SALICYLATE

Centrifuge run No.	Fraction (based on concentration of sodium salicylate)	Protein concentration, %	s_{20}	Protein molecularly dispersed, %
3	Soluble after dialysis of 8% against 1%	0.51	2.68	—
4	Ppt'd during dialysis of 8% against 1%	1.02	2.72	64
5	<i>AI</i> ** Ppt'd on dilution of 8% to 6.5%	0.20	—	—
6	<i>AI</i> Ppt'd on dilution of 6.5% to 5%	0.60	2.64	55
7	<i>AI</i> Ppt'd on dilution of 5% to 3%	0.99	2.61	88
8	<i>AI</i> Ppt'd on dilution of 3% to 1%	1.00	2.72	106
9	<i>AV</i> Ppt'd on dialysis of 1% against water	0.50	3.04*	101

* Four replicate values in poor agreement.

** Letters merely identify the series of fractions. (See Tables VII and X).

This was followed by a more complete fractionation in which fractions were obtained by direct dilution of the dispersion in 8% sodium salicylate. The concentrations of salicylate used and the results obtained are presented in Table V, Runs 5 to 9. All fractions were redispersed in 8% sodium salicylate regardless of the conditions of precipitation. The first fraction was very small, and gave no measurable results in the centrifuge. Obviously most, if not all, of this fraction was aggregated, and sedimented out before full speed was reached. The sedimentation constants for the next three fractions did not vary significantly, but as the percentage of the protein that was molecularly dispersed increased with increasing solubility of the fractions, there appeared to be definite differences among them. The final fraction, obtained by dialysing the 1% salicylate against water, was all molecularly dispersed but yielded a higher s_{20} value. The replicate values were in poor agreement, however, the final one being no higher than those for the other fractions.

The differences in the molecular dispersion of the first four fractions might be explained in various ways. The fact that there was no sharp break in aggregation between any two fractions indicated that there was overlapping of solubility, or that the molecularly dispersed portion was really the same in each fraction, but varied as a proportion of the total protein. Thus the molecularly dispersed protein in all fractions might be considered as gliadin. There were reasons for not accepting this explanation, however, chief among them being that the sedimentation diagrams for the molecularly dispersed protein were not typical of a homogeneous protein. Since later experiments yielded a more definite answer to this question, it need not be further discussed here.

The result obtained with Fraction AV led to a few runs being made on extracts of flour and wheat. The results had suggested that the crude gluten contained some protein material that sedimented faster than the main component. This was in agreement with the findings of Krejci and Svedberg on salt extracts of flour (14). The results with extracts in the present study failed to confirm this suggestion, but as it was obvious that much more work would need to be done to reach any acceptable conclusions, and as the protein involved was definitely not an important part of the gluten complex, these studies were abandoned in favour of detailed work on gluten.

EFFECT OF SALICYLATE CONCENTRATION ON DISPERSION

In earlier studies it had been determined that the amount of gluten dispersed was proportional to the sodium salicylate concentration used (18) and that this value was approximately the same whether the gluten was extracted with salicylate of the appropriate concentration or this concentration was obtained by dilution of a stock dispersion in 8% salicylate (26). These results suggested that the amount of protein molecularly dispersed in the first fractionation experiment might have been quite different had each fraction not been studied in 8% sodium salicylate.

A concentrated stock dispersion of gluten from ether extracted flour was prepared in 8% sodium salicylate. Aliquots were diluted to 6, 4, and 2% salicylate, and other aliquots made up to 10, 12, and 16%. The dilutions brought about varying amounts of precipitation, and the precipitates were removed before the dispersions were used in the ultracentrifuge. All preparations were subjected to the same conditions, and results are presented in Table VI. It should be noted that the difficulties in calculating concentrations from the sedimentation diagrams are greatly increased with the use of 12 and 16% salicylate solutions but that the increases in molecular dispersion as compared with the dispersion in 8% are undoubtedly significant.

The decrease in sedimentation constant is difficult to explain when considered together with later results obtained in detailed fractionation experiments. It seems possible, however, that the state of the *same* protein material is slightly altered by salicylate concentration, as it was found that there was a definite difference in partial specific volume of the gluten protein in 8 and 12% sodium salicylate. The differences in s_{20} are small, and it

TABLE VI

RELATION BETWEEN SALICYLATE CONCENTRATION AND MOLECULAR DISPERSION, ETHER EXTRACTED SOFT FLOUR GLUTEN

Centrifuge run No.	Salicylate concentration, %	Protein concentration, %	ζ_{20}	Protein molecularly dispersed, %
31	2	0.55	2.66	14
28	4	1.03	2.70	34
29	6	1.28	2.61	48
22	8	1.06	2.47	68
33	10	1.17	2.44	72
25	12	1.06	2.45	84
27	16	1.40	2.37	77

must be concluded that the material that was molecularly dispersed in each salicylate concentration behaved in essentially the same way.

The proportion of the protein molecularly dispersed varied directly with the salicylate concentration. The dispersions, as used in each concentration, were opaque but cleared rapidly in the ultracentrifuge. These results, considered in relation to those obtained earlier with a similar flour (18, Flour 1) lead to the conclusion that any specific concentration of salicylate under definite conditions, disperses a definite proportion of the gluten protein, but only a part of this protein is reduced to molecular solution. This is best illustrated in Fig. 2. At each salicylate concentration up to 8% the protein exists in three states: molecularly dispersed, partially dispersed, and non-dispersed, and the amounts in each of these states vary with salicylate concentration. The partially dispersed material, however, exists in many different degrees of aggregation, a fact more clearly brought out by later experiments.

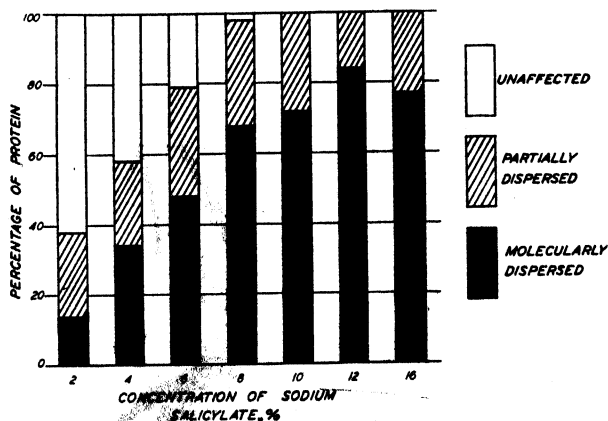


FIG. 2. Relation between concentration of sodium salicylate and gluten protein dispersion.

The portion of the protein partially dispersed in 2% sodium salicylate was all molecularly dispersed in 8% salicylate. This may be assumed from Fig. 2, and it is experimentally proved by results with Fraction AIV (Table V) because all of the protein partially dispersed by 3% salicylate was molecularly dispersed in 8%. Thus not only the protein that was molecularly dispersed in 2% salicylate, *but also that which was aggregated*, was molecularly dispersed in 8% salicylate, and gave essentially the same sedimentation behaviour. It is certain that the same effect is found with most of the protein partially dispersed in 4% salicylate, and with a portion of that partially dispersed in 6%.

Two important conclusions emerge from this experiment. First, there is no distinct break in the relation between protein solubility and salicylate concentration as would be expected if the gluten consisted of two or three distinct protein species. Second, the same protein material exists in entirely different forms under different solvent conditions. The results obtained with 2% salicylate in the present experiment and with Fraction AIV, Table V, make it impossible to consider the protein involved as an individual substance. Since all of this, and more, is included in what has been called "gliadin" and, since there is no sharp break in properties over the whole range of solubility, the results of the experiment must be considered as direct evidence against the existence of major individual protein species in gluten.

EFFECT OF WASHING, PRECIPITATION, AND CLARIFICATION ON GLUTEN

The presence in crude gluten of a soluble protein fraction that differs markedly from the gluten fraction in properties has been demonstrated beyond any question (18, 26). In the present work, it was considered that the removal of this fraction in preparation of the gluten dispersions would be advantageous. Preliminary work on the solubility of the gluten and non-gluten proteins suggested that the non-gluten was soluble in 0.25% sodium salicylate while the gluten protein was little, if at all, dispersed by this concentration. An attempt was made to remove the non-gluten fraction by using 0.25% sodium salicylate as the washing solution. This proved to be relatively ineffective because when washing was carried on long enough to remove the non-gluten protein, definite signs of surface denaturation of the gluten developed. When the normal time of washing, rate of flow, etc., were used, there was little difference in the washing effect of the salicylate and the 0.1% phosphate buffer commonly used. This is shown in Table VII, Runs 34, 40, and 41.

It was then suggested that the desired result could be obtained by preparing the dispersion in the usual way, then precipitating the gluten protein either by dilution to 0.25% salicylate or by salting out with magnesium sulphate at 20% of saturation. Both of these methods were tried and the precipitated protein redispersed in 8% sodium salicylate. The results are given in Table VII, Runs 37 to 39. The sedimentation constant was little affected, but the sedimentation diagrams were greatly altered and indicated that the extra handling had resulted in considerable denaturation. This is illustrated

TABLE VII

EFFECT OF WASHING, PRECIPITATION, AND PRELIMINARY CLARIFICATION ON BEHAVIOUR OF GLUTEN IN THE ULTRACENTRIFUGE. ETHER EXTRACTED MARQUIS FLOUR

Material	Washing solution	Precipitated by:	Run No.	Salicylate concentration, %	Protein concentration, %	s_{20}	Protein molecularly dispersed, %
Crude dispersion	0.1% phosphate	Not ppt'd	34	8	1.14	2.61	62
Crude dispersion	0.1% phosphate	Not ppt'd	40	8	1.19	2.59	59
Crude dispersion	0.25% salicylate	Not ppt'd	41	8	1.11	2.58	62
Crude dispersion	0.25% salicylate	Not ppt'd	43	12	1.09	2.51	71
Redispersion	0.1% phosphate	Dilution to 0.25% salicylate	37	8	1.28	2.58	—
Redispersion	0.1% phosphate	Dilution to 0.25% salicylate	39	8	1.31	2.51	—
Redispersion	0.1% phosphate	Salting out	38	8	1.15	2.71	—
Clarified dispersion	0.25% salicylate	Not ppt'd	44	12	1.10	2.49	80
Clarified dispersion	0.25% salicylate	Not ppt'd	45	8	1.12	2.62	75

in Fig. 3, which includes diagrams obtained with a crude dispersion (Run 34) and two precipitated and redispersed preparations (Runs 37 and 38). The shape of the diagrams made it difficult to determine concentrations and, therefore, the proportion of the gluten protein molecularly dispersed. The disadvantages resulting from denaturation certainly more than offset any

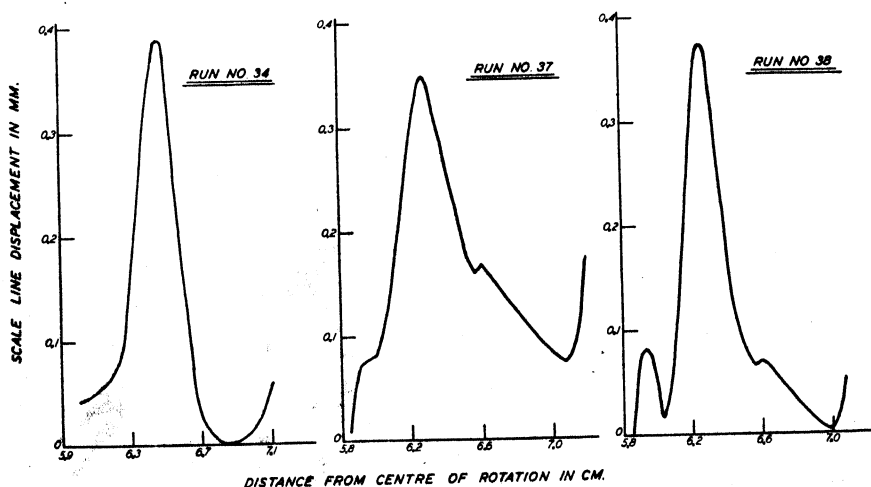


FIG. 3. Sedimentation diagrams of a crude gluten dispersion (run No. 34), gluten precipitated by dilution (run No. 37), and gluten precipitated by salting out (run No. 38). All runs made in 8% sodium salicylate solutions.

advantages resulting from the separation of the non-gluten protein, and as the mildest possible treatment of the gluten during precipitation and redispersion caused denaturation, this procedure was abandoned.

The rapid sedimentation of the aggregated material causing opacity of all dispersions, and the incomplete molecular dispersion even in 12% salicylate suggested that preliminary clarification of the preparations might remove most of the aggregated protein, and permit much more definite separation of the remaining material. The results of the first fractionation experiments indicated overlapping in degree of dispersion among the fractions, and this makes any definite conclusions as to the detailed make-up of the gluten more difficult to reach.

A preliminary clarification experiment had been carried out by centrifuging a crude dispersion for 18 hr. in a low-speed ultracentrifuge at 18,000 r.p.m., using the separation cell described by Svedberg and Pedersen (27, p. 152). This prevented the remixing of the heavy aggregated material with the clarified dispersion, which was recovered and used in ultracentrifuge and diffusion runs. The results showed that the sedimentation constant was unaltered (2.51) and the percentage of molecular dispersion increased but still far from complete. The mean diffusion constant was 5.30×10^{-7} *, but as it varied from 3.25 to 6.54 with distance from the position of the original boundary, and as the diffusion curve was far from normal (in the mathematical sense) it was obvious that this material was very inhomogeneous.

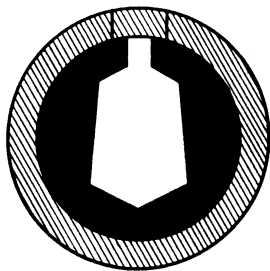


FIG. 4. End view of cell used for clarifying dispersions.

It had also been noted in this preliminary experiment that some, and probably most, of the sedimented material formed a tenacious layer at the bottom of the cell. It was therefore thought that large cells might be used to carry on this preliminary clarification and provide sufficient material not only for use in the ultracentrifuge but also for fractionation and chemical analysis. Two cells with their centre parts of Perspex were constructed, each having a capacity of approximately 4.5 cc. and the shape shown in Fig. 4. The solid material which was rapidly sedimented out collected at the bottom and remained there even during the stopping of the centrifuge. Since no photographic exposures were required, both cells could be filled with

* Diffusion constants are reported in units of 10^{-7} cm.²/sec.

protein dispersion and 7 to 8 cc. recovered after clarification. A preliminary run was carried out on gluten in 12% sodium salicylate. The material recovered was divided into two aliquots, one being dialysed against 12%, and one against 8% sodium salicylate. The dialysed solutions were both clear, and were used in ultracentrifuge runs. The results are given in Table VII, Runs 44 and 45. These show again that the sedimentation constants were unaltered (compare with Runs 34 and 43) but that the molecular dispersion was more complete than with unclarified material. The results also agreed with those obtained earlier in that there was less protein molecularly dispersed in 8% than in 12% sodium salicylate. It must be noted, of course, that, as a percentage of the total gluten protein, there was a slight decrease in the amount molecularly dispersed, because some of the more soluble portion of the gluten was retained with the sediment. It was impossible to correct these values because the amount of the soluble protein so retained could not be accurately determined.

ANALYSIS OF, AND DETERMINATIONS ON, FRACTIONS FROM A CLARIFIED DISPERSION

A concentrated dispersion of gluten in 12% sodium salicylate was prepared from ether extracted Marquis flour and used in a centrifuge run (after dilution of an aliquot) for fractionation and in preparing a bulk of clarified material using the cells described in the preceding section. The clarified dispersion was also used for a centrifuge run and for fractionation. Samples of crude gluten, of crude gluten dispersed in sodium salicylate and precipitated at 20% of saturation with magnesium sulphate, of the clarified dispersion, and of each fraction, were hydrolysed in 20% hydrochloric acid, and amide nitrogen was determined.

The sediment which formed during clarification in the low-speed ultracentrifuge exhibited very distinct characteristics. It was sedimented in two general layers which gradually merged into each other. At the bottom of the cell was a very tough opaque substance, while at the top of the solid layer the sediment was a clear jelly. The jelly was readily redispersed in 8 or 12% sodium salicylate, but the opaque material was not. Some of the aggregates heavy enough to be sedimented thus yielded a clear dispersion. There appeared to be a definite point at which the sedimentation stopped *for these particular conditions*, as recentrifuging the clarified dispersion did not yield any further sediment. This was undoubtedly an equilibrium effect which would have been altered by varying the conditions.

It was certain that if this sediment contained material distinctly different from that in the clarified dispersion, it should be low in amide content (18). Sediment from several runs was collected and in one instance the opaque sediment separated from the clear jelly. These samples were also hydrolysed and analysed for amide nitrogen.

Fractionation of both the clarified and crude dispersions was carried out by diluting the salicylate first to 4.5%, then to 2.5%, and finally to 0.25%. The resulting fractions were redispersed in 12% salicylate. The first fraction

from the crude dispersion was clarified in the low-speed ultracentrifuge and both the clarified portion and the sediment analysed for amide nitrogen. The other fractions all yielded clear solutions on redispersion, and aliquots of the protein were used for analysis.

The results of all amide analyses together with the percentage of the total gluten nitrogen involved, and the midpoint of the fraction (18, 26) are presented in Table VIII. These results confirm in every respect those obtained in earlier work. The opaque sediment (most insoluble fraction of the gluten) was the lowest in amide, as expected, and clarification raised the amide content of the dispersed protein. Every value obtained fits excellently into the relation between gluten fractions and amide content determined in earlier work (Fig. 5). The low amide values obtained for the most soluble gluten fraction in that work (18) were not encountered here because fractionation was confined to the true gluten protein.

TABLE VIII

EFFECTS OF CLARIFICATION AND FRACTIONATION ON THE AMIDE CONTENT OF GLUTEN PROTEIN

Fraction	Percentage of total gluten nitrogen	Amide nitrogen content, %	Midpoint of fraction, % of gluten nitrogen
Crude gluten (non-dispersed)	100	22.3	—
Gluten dispersion, precipitated*	92	23.4	46
Gluten dispersion, precipitated*	93	23.3	46.5
Gluten dispersion, clarified in 12% salicylate*	65	25.0	60
Gluten dispersion, clarified in 8% salicylate*	58	25.1	63
Gluten dispersion, clarified in 8% salicylate*	57	25.1	62.5
Sedimented during clarification in 12%	27	19.7	13.5
Sedimented during clarification in 8%	34	20.5	17
Opaque sediment only	19	17.8	9.5
Fraction I from crude dispersion	46	20.1	23
Fraction I from clarified dispersion	5	—	—
Fraction I sedimented during clarification	22	19.0	11
Fraction II from crude dispersion	18	25.0	58
Fraction II from clarified dispersion	26	23.2	45
Fraction III from crude dispersion	26	27.2	78.5
Fraction III from clarified dispersion	35	26.1	75.5

* *Precipitated with magnesium sulphate at 20% of saturation, after clarification in low-speed ultracentrifuge.*

The results obtained with the protein sedimented out offer perhaps the best answer yet found to the suggestion that fractions obtained by precipitation may be adsorption complexes (3). Such sediment forms a fraction that is now shown to vary in composition with the amount thrown down. Since the analysis of these "fractions" obtained by centrifuging agrees exactly

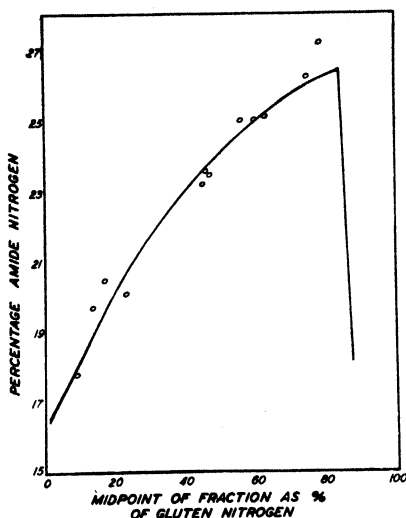


FIG. 5. The amide content of gluten as affected by position of fraction in the gluten complex. Points obtained in present experiment, solid line in earlier work (18).

with those obtained by precipitation it seems both unnecessary and incorrect to assume that adsorption of two (or a few) protein species in different proportions produces the type of fraction isolated by McCalla and Rose (18).

The results obtained in centrifuge runs with the crude and clarified dispersions, and with the three fractions of the latter, are presented in Table IX.

TABLE IX

SECOND FRACTIONATION EXPERIMENT, MARQUIS FLOUR. ALL RUNS IN 12% SODIUM SALICYLATE

Centrifuge run No.	Fraction No.	Material	Protein concentration, %	s_{20}	Protein molecularly dispersed, %	D_{20}
43	—	Crude gluten dispersion	1.09	2.52	71	—
44	—	Gluten dispersion clarified at 12,000 r.p.m. for 24 hr.	1.10	2.49	80	—
48	BI	Fraction soluble in 12% salicylate, ppt'd by 4.5%	1.19	2.56	50*	—
46	BII	Fraction soluble in 4.5% salicylate, ppt'd by 2.5%	0.99	2.63	93**	3.30
47	BIII	Fraction soluble in 2.5% salicylate, ppt'd by 0.25%	0.92	2.45	102	4.89

* Sedimentation diagrams asymmetrical and base-lines poor.

** Sedimentation diagrams at 60 and 100 min. after starting were asymmetrical, but indicated 100% recovery of the protein. Later diagrams were more symmetrical, but percentage recovery less, falling to 77% at 220 min.

These results confirm those presented in Table V (obtained using unextracted soft wheat flour) except that the proportion of the total gluten molecularly dispersed is somewhat less. The most soluble fraction, however, was completely dispersed as was the somewhat comparable fraction (AIV) of the earlier experiment. The results obtained with the second fraction (Run 46) are of particular interest. Complete molecular dispersion was indicated by the first two diagrams, but only 77% of the total gluten was so dispersed at the end of the run. This apparent decrease, however, was not accompanied by the formation of any measurable components. It appears that this protein material gradually sediments, and this particular behaviour is evidence in support of the thesis that the gluten complex varies regularly in properties.

Diffusion constants (D_{20}) were determined for the two more soluble fractions, and are also reported in Table IX. The difference in these two values shows that the mean molecular sizes (or shapes) of these two fractions are much more different than is indicated by the slight variation in sedimentation constants. This difference is partially accounted for by the presence of aggregates in the less soluble fraction, but this is inadequate to explain the difference between D_{20} values of 3.30 and 4.89. If the molecularly dispersed particles of Fraction II were of the same size and shape as those of Fraction III, the diffusion of these particles alone would have resulted in a higher D_{20} value than was obtained for this fraction. Since the aggregated portions of Fraction II must have contributed something to the weight-average D_{20} , there can be no doubt that the molecularly dispersed particles of the two fractions were definitely different in character. This is the most unequivocal evidence of difference in the size or shape of fractions of the gluten protein usually called gliadin, since both of these fractions are part of this protein.

It is of interest to note that the diffusion constant of a clarified preparation of gluten was lower in 12% sodium salicylate than in 8%. This is the reverse of what would be expected from the sedimentation results which showed a greater proportion of the gluten molecularly dispersed in 12%, but since sedimentation rate is lower in 12%, it seems likely that some common factor may affect both of these determinations. The factor involved may be a combination between the salicylate and the protein, a possibility also suggested by preliminary electrophoresis experiments.

MAIN FRACTIONATION EXPERIMENT

Following the experiments reported in Table VII, considerable work was carried out on fractions of gluten obtained in various ways. It was finally decided to make a thorough investigation of four fractions of gluten from ether extracted Marquis flour obtained by salting out from a clarified dispersion with magnesium sulphate. Clarification in the low-speed ultracentrifuge sedimented out approximately 18% of the total gluten protein. Magnesium sulphate solution was added to the clarified dispersion to make the solution 3% saturated with respect to the salt. The precipitate was centrifuged and washed; and the solution made up to 5% of saturation with

magnesium sulphate. The process was repeated with 8% and 20% of saturation, each precipitate being centrifuged, washed, and recentrifuged. Approximately 7% of the protein remained unprecipitated, but this is non-gluten in nature (18). Each fraction was redispersed in 12% sodium salicylate.

Each fraction was used in sedimentation velocity, sedimentation equilibrium, and diffusion studies. The summarized results are given in Table X together with the results for the most soluble fraction of the second fractionation experiment (Table IX).

TABLE X

SUMMARIZED RESULTS OF MAIN FRACTIONATION EXPERIMENT, ETHER EXTRACTED MARQUIS FLOUR. ALL RUNS IN 12% SODIUM SALICYLATE

Fraction	Conc. of MgSO_4 , % saturation	Total gluten protein in fraction, %	Protein concentration, %	s_{20}	Protein molecularly dispersed, %	D_{20}	Weight-average molecular weight		f/f_0
							(a)*	(b)**	
C I	3	37	0.48	2.57	—	2.14	1,750,000	—	1.27†
C II	5	9	0.54	2.51	—	3.13	322,000	—	1.52†
C III	8	12	0.52	2.43	89	4.10	102,000	—	1.71†
C IV	20	24	0.54	2.36	100	4.83	45,000	43,000	1.90†
B III	—***	26	0.92	2.45	102	4.89	—	44,000	1.94††

* (a) calculated from equilibrium data using formula $M_w = \frac{RT}{(1 - V\rho)^2} \cdot \frac{dc}{dx} \cdot \frac{1}{x.c.}$

** (b) calculated using formula $M = \frac{RTs_{20}}{D_{20}(1 - V\rho)}$ and assuming that values determined at 24° C. are not significantly different from those determined at 20° C.

*** Obtained by dilution, second fractionation experiment.

† Obtained from M_w and D_{20} data.

†† Obtained from s_{20} and D_{20} data.

There is a definite downward trend in the sedimentation constant with increasing solubility of the fractions. This is the only experiment that yielded such definite results, but it was by far the most carefully controlled, e.g., each dispersion was dialysed against several changes of sodium salicylate before being used for any determination, and every effort was made to have all conditions as constant as possible. These results show that the molecularly dispersed protein in the four fractions was not uniform even in sedimentation behaviour and it has already been shown that such protein did not give uniform diffusion results. The amount of protein molecularly dispersed could not be calculated for the first two fractions because the base-lines of the diagrams could not be established. The results with the more soluble fractions are in excellent agreement with those for corresponding fractions in the second fractionation experiment.

Diffusion results show a progressively higher rate with increasing solubility, but the numerical differences in the results are by no means comparable to the differences in molecular weight as determined with the equilibrium cen-

trifuge. Since only part of the total protein of the first three fractions was included in the sedimentation velocity determinations, diffusion and sedimentation constants do not apply to the same material. The weight-average molecular weight of the most soluble fraction (*C IV*) is the same within the limits of experimental error, whether calculated from sedimentation velocity and diffusion data, or from equilibrium data. This confirms the conclusion that this fraction is all molecularly dispersed. Since none of the other fractions is completely molecularly dispersed, the s_{20} values cannot validly be used for calculating molecular weights. There is no question but that decreasing solubility is accompanied by increasing proportions of the protein in aggregated form. The "molecular weights" for these fractions are, therefore, not truly weights for individual molecules.

The calculation of frictional ratios (f/f_0 , Table X) gives information as to the shape of the molecules (27, p. 10). Because there were varying proportions of aggregates, it does not, unfortunately, give information as to the relative shape of the *molecularly dispersed* portions of the four fractions so it is impossible to draw conclusions as to this very important factor. The results obtained show that the mean frictional ratio is greatest for Fraction *C IV* and least for Fraction *C I*, a result the opposite to that which would be expected if Kuhlmann's conclusions were correct (15). It is also the opposite to that expected from a priori reasoning based on most explanations of the peculiar properties of gluten.

This result, however, does not necessarily mean that the basic molecules of Fraction *C I* are shorter than those of Fraction *C IV*. Fraction *C I* contains a large proportion of aggregates, and these may well be aggregated in bundles that have diameters much greater than that of the individual molecule. These bundles could quite conceivably be made up of molecules that individually have a higher frictional ratio than those of Fraction *C IV*, but collectively have a lower one. The present results are inadequate to settle this question, and further speculation appears unwarranted and unprofitable.

The inhomogeneity of fractions as prepared in this study has already been mentioned. The fractions are quite arbitrarily prepared and are not considered as representing any true fraction of the original material. The validity of this consideration is shown by the detailed results of equilibrium determinations presented in Table XI and by the diffusion diagrams in Fig. 6. The diffusion "constants" calculated from different parts of these diffusion diagrams were not constant for any of the fractions. The variability found was as follows: Fraction *C I*, 0.79 to 3.20; Fraction *C II*, 1.72 to 3.46; Fraction *C III*, 2.72 to 4.56; Fraction *C IV*, 4.00 to 6.12; recombined fractions, 1.33 to 4.87. The calculated weight-average diffusion constant for the four individual fractions was 3.32, while the experimental weight-average for the recombined fractions was 3.36. The latter value was obtained by recombining the four fractions in proportion to the total protein of each fraction, and carrying out a diffusion determination.

TABLE XI

DETAILED RESULTS OF SEDIMENTATION EQUILIBRIUM RUNS, MAIN FRACTIONATION EXPERIMENT

Fraction	x^* , cm.	Concentration of protein, %	$\frac{dc}{dx}$	Molecular weight	Fraction	x^* , cm.	Concentration of protein, %	$\frac{dc}{dx}$	Molecular weight
C I	4.87	0.410	0.185	470,000	C II	5.58	0.513	0.092	163,000
	4.90	0.418	0.243	600,000		5.60	0.515	0.102	179,000
	4.95	0.433	0.353	830,000		5.65	0.521	0.126	216,000
	5.00	0.454	0.486	1,080,000		5.70	0.528	0.155	260,000
	5.05	0.485	0.735	1,520,000		5.75	0.537	0.175	287,000
	5.10	0.533	1.215	2,280,000		5.80	0.546	0.190	304,000
	5.15	0.616	2.083	3,320,000		5.85	0.557	0.233	362,000
	5.16	0.641	3.000	4,590,000		5.90	0.570	0.306	460,000
Weight-average molecular weight				1,750,000	Weight-average molecular weight				322,000
C III	4.81	0.447	0.312	72,100	C IV	5.59	0.497	0.219	39,200
	4.85	0.460	0.324	72,200		5.60	0.499	0.223	39,700
	4.90	0.477	0.341	72,500		5.65	0.510	0.228	39,300
	4.95	0.495	0.370	75,000		5.70	0.522	0.238	39,800
	5.00	0.514	0.405	78,300		5.75	0.534	0.248	40,100
	5.05	0.536	0.469	86,100		5.80	0.547	0.262	41,000
	5.10	0.563	0.613	106,000		5.85	0.560	0.286	43,400
	5.15	0.603	0.995	159,000		5.90	0.576	0.354	51,800
Weight-average molecular weight				102,000	Weight-average molecular weight				45,000
	5.18	0.638	1.320	198,000		5.94	0.596	0.480	67,400

* Distance from centre of rotation.

These results indicate that Fraction C I was the most inhomogeneous, while Fraction C IV was the least, as would be expected. That there was overlapping between Fractions C I and C II, and between C II and C III, is not surprising, since these three fractions each contained both molecularly dispersed and aggregated material. Since all of Fraction C IV was molecularly dispersed, however, the overlapping between Fractions C III and C IV, although not great, is probably a reflection on the method of fractionation, indicating that the separation of these fractions is not as sharp as might be desired. That the overlapping of these two fractions is not great, however, is definitely shown by the detailed molecular weight results presented in Table XI.

Equilibrium with Fractions C III and C IV was established at 4000 r.p.m. and for Fractions C I and C II at 1200 r.p.m. In each run, about eight days were required to establish equilibrium. Had Fraction C IV been subjected to a higher centrifugal force, it seems certain that the inhomogeneity in molecular weight results would have been greater. In the present experiment, the concentration of protein at the top of the column was 0.497% and at the bottom 0.596%, a comparatively small difference that would have been decidedly increased with increased speed. Such increased speed would probably have reduced the minimum value of 39,200 for the molecular weight, and increased

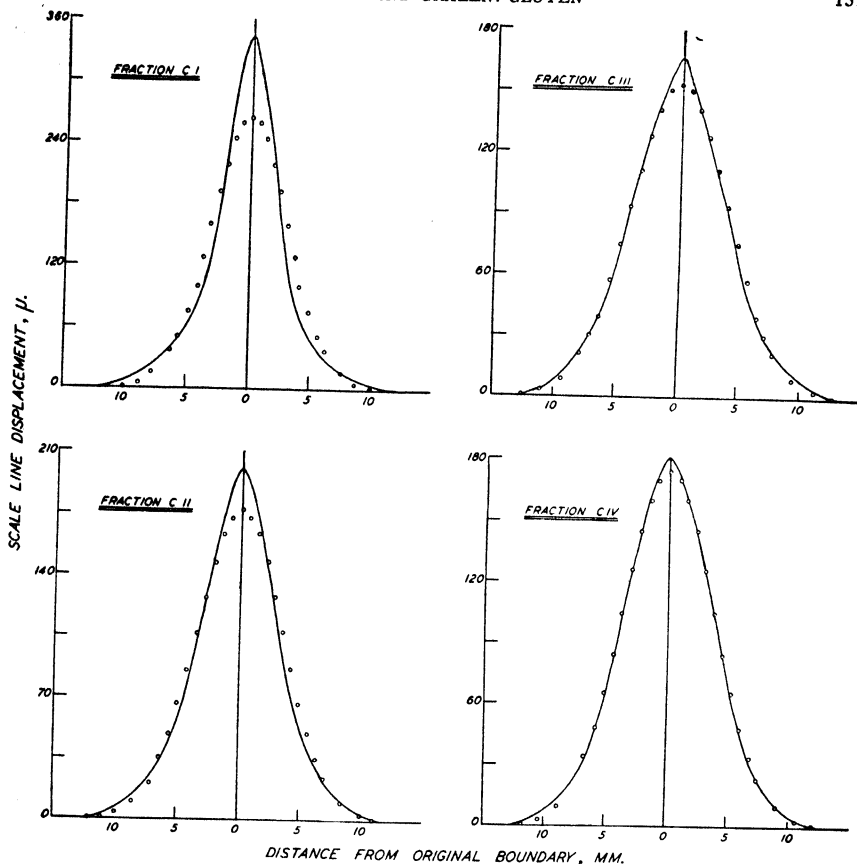


FIG. 6. Diffusion diagrams obtained with the four gluten fractions of the main experiment. Points represent the theoretical, normal curve, the solid line is the experimental curve.

the maximum value. It seems possible that the true minimum is the Svedberg unit of approximately 35,200, although there is little evidence that this unit is significant for proteins that are not homogeneous, globular, and crystalline.

Kraemer (27, pp. 342-353) has discussed the methods of calculating molecular weights for polydisperse systems, and states that the characterization of the degree of heterogeneity is difficult, because sedimentation equilibrium does not distinguish between a mixture of a few molecular species of markedly different weight and a mixture of a great many species differing by small increments in weight. He suggests however, that the non-uniformity coefficient β , which is a parameter defining the spread of the distribution of particle weight, is useful in characterizing the molecular weight of polydisperse systems. The application of this calculation to the present data has been discussed

with Kraemer*, and the results of the calculations are presented in Table XII. Before the values for M_z and β were calculated, the sedimentation equilibrium data were plotted as $\log c_x$ against x^2 (27, p. 347). The resulting curves are given in Fig. 7. The smoothed data were used in calculating M_z and β .

TABLE XII

AVERAGE MOLECULAR WEIGHTS AND DISTRIBUTION PARAMETERS FOR MAIN FRACTIONATION EXPERIMENT

Fraction	M_w	M_z	M_w/M_z	β
<i>C I</i>	1,750,000	11,900,000	6.80	1.97
<i>C II</i>	322,000	2,840,000	8.82	2.07
<i>C III</i>	102,000	432,000	4.23	1.72
<i>C IV</i>	45,000	171,000	3.72	1.64

The results, as given in the curves, apparently agree with those of the diffusion experiments, and with the degree of heterogeneity as shown by the detailed equilibrium results given in Table XI, although Kraemer points out that interpretation of these curves is difficult. Fraction *C IV* appears to be the most nearly homogeneous, and Fraction *C I*, the least. None of these fractions, however, is homogeneous.

The non-uniformity coefficients are very difficult to reconcile with all these other results. Even Fraction *C IV* appears to be very heterogeneous, almost as much so, in fact, as any of the examples given by Kraemer (27, p. 353). The results for Fraction *C II* appear to be out of line with those for the other fractions. This β value, however, can be questioned, because a much greater degree of smoothing was required with the graph for this fraction than for any of the others.

At the moment, no adequate explanation or interpretation of these results can be given. It seems possible that some factor not encountered with other material may be important with these gluten fractions. It would not be surprising to get high β values for the fractions containing both molecularly dispersed and aggregated protein. It is surprising, however, to get such a high value with Fraction *C IV*. One conclusion can be reached, however, and that is that these results add important evidence in support of the belief that gluten is an extremely heterogeneous system. The results for Fraction *C IV* are again, as in earlier experiments, the most convincing because aggregates do not appear to be present in this fraction. If the β value of 1.64 truly represents the particle distribution of molecularly dispersed protein, then this fraction is more heterogeneous than the other results indicate.

Recently, Gralén (10) has discussed polydispersity as determined from diffusion measurements, and proposed a new non-uniformity coefficient, γ which is related to β by: $\gamma = \beta/\alpha$, α being a constant which is less than 3

* Kraemer, Elmer O., private communication. At the time this discussion was written, communication with the junior author was impossible. The senior author, therefore, assumes all responsibility for the discussion of β values and the conclusions reached from this discussion.

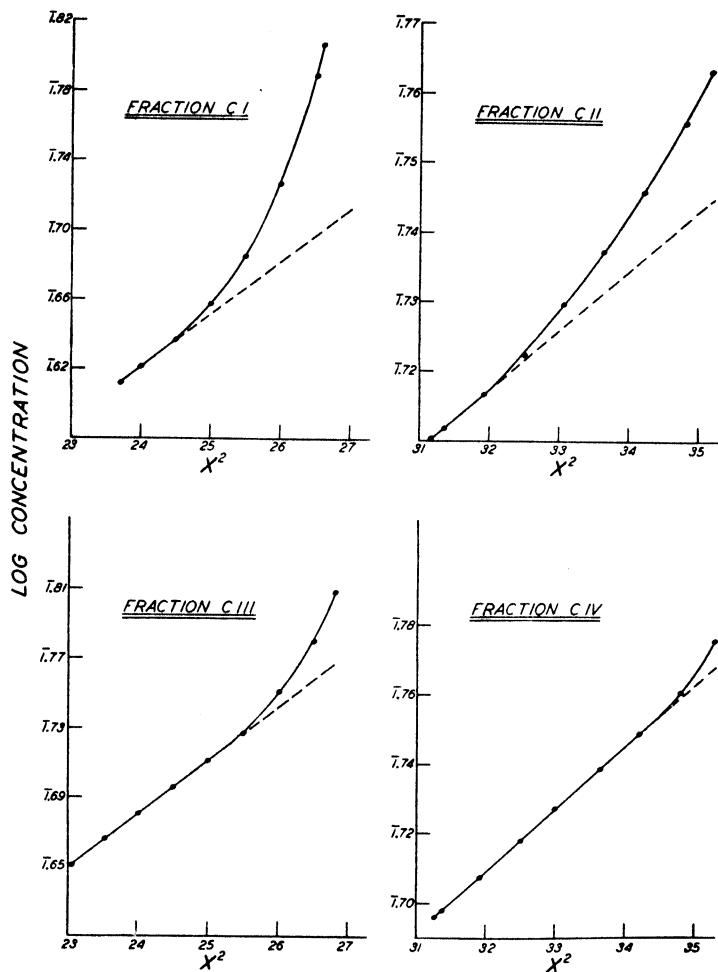


FIG. 7. Curves obtained by plotting $\log c_*$ against x^2 . Data from equilibrium runs on the four fractions of the main experiment. The solid line is the experimental curve; the deviation from a linear relation is also indicated.

and for very slender molecules approaches 1. He gives $\gamma = 0.7$ for a preparation of gluten protein in 8% sodium salicylate, but unfortunately does not give α . The α for dinitrocellulose was estimated as 1.2 and it seems likely that the value for gluten would be considerably higher since the frictional ratios indicate gluten molecules to be less thread-like than are cellulose and its derivatives. If the α for gluten is equal to 2 or more, the β calculated from Gralén's data would be in fair agreement with that for Fraction C IV.

It seems unlikely that the inhomogeneity of Fraction C IV is brought about by the presence of multiples of any basic unit. The increase in molecular

weight must be gradual because all of the protein appears as one component in the high-speed ultracentrifuge. This component must be made up of portions of markedly different weight, but similar behaviour. The most likely explanation is that the length of the molecules varies considerably, affecting weight decidedly but sedimentation behaviour only slightly. There is considerable speculation attached to this conclusion, but there is also considerable fact to support it, and many difficulties in explaining it on any other basis. The possibility of variable hydration instead of variable length of molecules cannot be dismissed. Svedberg and Pedersen (27) discuss this problem but no final conclusion is reached. As a matter of fact, there are too few data on the physical properties of such proteins as gluten to enable one to reach any very definite conclusions. It has been suggested that such protein in salicylate dispersion may be a mixture of various degradation products, but it is very difficult to accept this suggestion since gluten with all the properties of the original material can be recovered from the dispersion by salting out. It is our belief that the characteristics of such a system as gluten differ so fundamentally from the properties of the homogeneous globular proteins that much more must be done before the divergence in opinion and discrepancy in results can be explained.

SUPPLEMENTARY EXPERIMENTS

Two further experiments were carried out in an effort to determine whether the results obtained with gluten dispersions prepared in sodium salicylate solutions were unique for these conditions.

Arrhenius (2) had made a study of gliadin prepared in 60% ethyl alcohol and concluded it was homogeneous with a molecular weight of 28,300. Later he carried out further experiments* which showed some of the earlier conclusions to be in error. Originally he had reported a sedimentation constant of 2.0×10^{-13} but more recently had determined it to be 2.6×10^{-13} . A gliadin preparation (about three months old) from Swedish patent flour in 60% alcohol was supplied by Arrhenius and was used as follows:

An aliquot was dialysed against 8% sodium salicylate for four days with frequent changes of solution. At first, a precipitate formed and rapidly settled, but most of this precipitate redispersed as dialysis proceeded. The final solution was nearly clear and contained no trace of alcohol. This dispersion was then used in the high-speed ultracentrifuge and for diffusion. The centrifuge run gave results that agreed with those already obtained, since the sedimentation constant was 2.45 and the diagram similar to those obtained earlier. Only 68% of the protein was molecularly dispersed, however, indicating considerable aggregation. The mean diffusion constant was 4.72, but this value varied widely with distance from the original boundary, indicating decided inhomogeneity. Thus, the studies on "gliadin" yield results in excellent agreement with those obtained with preparations in sodium

* *Unpublished results.*

salicylate, and show the "gliadin" to be very inhomogeneous. That the age of the preparation was responsible for such inhomogeneity is not, it is thought, a satisfactory explanation.

These results, however, were also obtained using sodium salicylate as dispersing agent during the tests. An experiment in which gluten was dispersed in acetic acid did not give acceptable results because the solvent failed to overcome the electrostatic charge on the protein. This resulted in a low S_{20} value and an asymmetrical diffusion diagram. This effect could not be overcome by adding salt because under these conditions the acid lost most of its dispersing power. The acetic acid molecularly dispersed 77% of the total gluten protein, a result not affected by the electrostatic effects.

In the meantime, Sandstedt* had supplied the writers with details regarding the dispersing solution used by Sandstedt and Blish (23) in numerous experiments. Gluten was prepared from ether extracted Marquis flour and dispersed in 0.1 *M* acetic acid. Dispersion was rapid, and an opaque solution formed. To this was added ethyl alcohol to make the solution 60% alcohol, and finally this solution was dialysed against a solution of 60% alcohol which was *M*/40 with respect to acetic acid and *M*/20 with respect to sodium acetate. This solvent was the same as that described by Sandstedt, but the procedure in preparation was quite different*. It involved the least mechanical manipulation of the gluten, and yielded the highest proportion of dispersed gluten obtained with any procedure tried. Even so, there was considerable precipitate formed, and eventually only about 50% of the total protein was included in the dispersion studied. The use of a colloid mill for preparing the dispersions (as described by Sandstedt) was definitely not satisfactory because the excessive mechanical treatment causes much more denaturation than that already shown to be very deleterious to the quality of the gluten.

The dispersion was not successfully used in the ultracentrifuge, but excellent results were obtained in a diffusion study. The all-glass cell (20) was used because of the alcohol, and the results showed that the electrostatic effect was removed, indicating that *M*/20 sodium acetate overcame the difficulty encountered with acetic acid alone. The mean diffusion constant was 4.44×10^{-7} , in excellent agreement with results for similar material in sodium salicylate. There was great variability, however, in different parts of the cell, the value near the top of the diagram being 2.82 and that near the bottom, 6.52. Thus, the gluten protein was fully as inhomogeneous in this solvent as in salicylate.

One further experimental result is of interest in connection with what has been considered protein "purity". Usually "gliadin" preparations are purified by chemical methods. Blish (3) considers that these methods can no longer be considered satisfactory as it is certain that changes in the physical properties of the gluten take place when alcohol is used at any stage of the preparation.

* *Private communication.*

Determinations of the nitrogen content of the protein in Fractions *C I* and *C IV* (Table X) were made. The values obtained, 17.60 and 17.87, are fully as high as those obtained for any "purified" preparations (6) and higher than many values reported. This is particularly true for Fraction *C I* which in classical terminology is glutenin. The only purification used for these preparations was a preliminary clarification before the fractions were produced.

Discussion

The results obtained in this study are not in agreement with those of some other workers. Krejci and Svedberg (13) found the molecular weight of gliadin to be 27,000 in alcohol and aqueous solutions, and concluded that the gliadin was not homogeneous. The work of Arrhenius has already been mentioned, and his earlier molecular weight values (2) confirmed those of Krejci and Svedberg. At present no explanation can be offered for these results but later determinations made by Arrhenius agree much better with those of the present study. Sodium salicylate, however, has a much less deleterious effect on the gluten than have most other solvents (7, 22) and the results of the present study are much more comprehensive than any others previously reported. In fact, the scope of many experiments carried out with gliadin or similar proteins does not permit anything like a complete estimation of the properties and make-up of such proteins being made.

In contrast to the ultracentrifuge results, however, the diffusion results reported here agree very well with those of Lamm and Polson (16, 20). Their gliadin was definitely inhomogeneous, and the fractions had markedly different diffusion constants. Only the most soluble fraction, representing about 8% of the gliadin (probably less than 4% of the whole gluten) was homogeneous, and inhomogeneity was most marked in the least soluble fraction. The results of the present study are of the same type, but they apply to the whole gluten. It is also very significant that the actual values for the diffusion constant of the more insoluble fractions (not included in Lamm and Polson's fractions) are much lower than any obtained by these investigators.

Time did not permit the carrying out of more detailed fractionation, but it seems almost certain, when the results reported in this paper are considered with those of Lamm and Polson, that a fraction having a higher diffusion constant than any prepared in the present study could have been obtained had fractionation been carried further. It is quite possible that a small soluble fraction would have a molecular weight of approximately 35,000, since, assuming the same sedimentation constant as for Fraction *C IV*, the diffusion constant necessary to give this molecular weight would be 6.3, a value lower than the maximum obtained by Lamm and Polson (16) and approached by the highest diffusion values obtained with Fraction IV. That 35,000 is approximately the minimum weight of the gluten protein is supported by the sedimentation equilibrium results in this study, since the 39,000 obtained is undoubtedly higher than the true minimum molecular weight.

Whether this fact has any importance for such a protein system as this may be questioned.

It is, perhaps, not out of place here to comment on work carried out with zein (28), secalin (1), and hordein (21). These proteins are similar to the so-called gliadin and results obtained with them should be of value in interpreting those of the present study.

Watson, Arrhenius, and Williams (28) obtained a mean molecular weight of 40,000 for zein, but isolated a fraction with a molecular weight of 35,000. The zein preparation was found to be polydisperse. It would appear, therefore, that these results are in entire agreement with those of this study.

Secalin was studied by Andrews (1), who found the diffusion constant to be 4.3 at 20° C. and estimated the molecular weight as 40,000. The formula used for calculating the diffusion constant, however, simply assumes the diagrams to be normal curves and therefore does not give any information on the homogeneity of the protein. That the assumption is unjustified is shown by diffusion studies with similar proteins (16, 20, 28). The calculation of the molecular weight is also questionable, since the diffusion and sedimentation constants will not apply to exactly the same material, unless all of the secalin were included in the sedimentation calculations, which seems very unlikely. Finally, the amount of impure secalin recovered is a very small part of the rye protein, and after purification, the preparations studied represented not more than 10%, and probably less, of the whole protein complex. The results of the present study show that generalization of conclusions based on such preparations is unjustifiable.

Hordein has been prepared by Quensel and Svedberg (21), in the same way as gliadin is usually prepared. No evidence of polydispersity was found, while $s_{20} = 2.0$, $D_{20} = 6.5$, and molecular weight = 27,500, assuming the same partial specific volume as for gliadin. These results are, therefore, in agreement with those of Krejci and Svedberg (13) for gliadin.

The occurrence of molecular weights of approximately 40,000 to 45,000 for zein (28), for gliadin in urea and alcohol solutions (5), for secalin (1), and for a gluten fraction in sodium salicylate in the present study would seem to be quite fortuitous. It is quite likely that some factor leading to the separation of approximately equal fractions of the total protein complex, e.g., the use of 60 to 70% alcohol in most preparations, has been responsible for the similarity in molecular weights as given above. It should also be noted that the method used by Burk (5) gives a number-average molecular weight that will differ considerably from the weight-average molecular weight of a heterogeneous dispersion.

An intermittent discussion of the constitution of gluten has been carried on for several years (3, 11, 15, 18, 23, 25, 26) and it is obvious that no generally accepted conclusions have been reached. There would seem to be no doubt, however, that previous work has shown that experimental evidence is incompatible with the idea of the existence of classical gliadin and glutenin.

In the present experiments, evidence of inhomogeneity is not based on the appearance of components on centrifuging, but it is none the less convincing. For this reason, conclusions regarding homogeneity of any of the seed proteins discussed above may be quite erroneous if a single component is taken as indicating homogeneity. All detailed diffusion results show these proteins to be polydisperse.

It is believed that the present results add the most convincing evidence yet obtained against the existence of two definite protein species. None of the results gave evidence of sharp breaks in properties of the whole protein complex. It still cannot be stated without qualification that *no* major protein species exist in gluten because it has been impossible to study the molecularly dispersed protein of the less soluble fractions free from aggregates. It may be that such isolated molecularly dispersed material cannot be prepared from these fractions, but if it could, it would be possible to determine definitely what differences exist in the properties of such protein from the different fractions. The most promising procedure would appear to be a study of fractions clarified for relatively long periods in the separation cell (27) because, if the aggregates could be sedimented, the remaining dispersed protein from the various fractions could be compared. It seems likely that the molecules would be progressively longer with decreasing solubility.

Whether or not this is true, the physical properties of the various fractions studied vary enormously. It is believed that such variability cannot be explained by assuming that gluten contains three or four protein species, appearing in varying proportions in the different fractions. The results appear to be best explained by the hypothesis (18) that the gluten protein is a protein system, which varies progressively and regularly in both chemical and physical properties. All the results are compatible with this hypothesis and many are difficult to explain on any other basis.

Acknowledgments

This extensive study would have been impracticable without the support of many persons and the financial assistance of several organizations. The authors wish particularly to thank Professor The Svedberg for placing the facilities of his laboratory at their disposal, and for his interest and help. Expenses incurred at Uppsala were defrayed by grants from the Rockefeller Institute and the Wallenberg Foundation. Financial assistance was obtained from the National Research Council of Canada, while a grant from the Searle Grain Company, Winnipeg, Man., covered travelling and incidental expenses of the senior author. To the officials of all these organizations, the authors express sincere thanks.

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STUDIES ON THE METABOLISM OF CEREAL GRAINS

I. THE OUTPUT OF CARBON DIOXIDE BY WHEAT GRAINS DURING ABSORPTION OF WATER AND GERMINATION¹

BY WILLIAM LEACH²

Abstract

The course of respiration during the early stages of germination is recorded for a number of varieties of bread and *durum* wheats. Germination is marked by three consecutive respiratory stages which are characterized by the rate at which the acceleration in carbon dioxide output occurs. These stages are (a) a slow rate of acceleration, (b) an increased rate followed by a decreasing rate, and (c) a final uniform and relatively high rate. Water absorption rate under the experimental conditions used does not appear to affect these respiratory stages. The infection of germinating grains by fungi reduces their respiration. Possible physiological explanations of the respiration stages are discussed.

Introduction

The effects of various factors on the respiratory activity of wheat have been the subject of a considerable amount of investigation. This has been due to the fact that respiration with its associated release of energy in the form of heat appears to be one of the primary causes of spoilage of grain during storage.

Two comparatively recent contributions in this field, both of which are primarily concerned with the practical standpoint of grain storage, are those of Bailey and Gurjar (1) and Larmour, Clayton, and Wrenshall (2). Bailey and Gurjar discuss the various aspects of the respiratory process in considerable detail and in addition give a comprehensive view of previous literature bearing upon the subject of wheat respiration. Their own investigations deal with the influence of water content and other factors on the carbon dioxide production of wheat under conditions of bulk storage. Larmour and his collaborators also examine the effect of water content on carbon dioxide output, but advance the opinion that the carbon dioxide and heat produced by wheat during storage are largely the result of the activity of fungi that infect the stored grain. They describe various treatments to reduce or inhibit this fungus activity.

In the present work are described the results of a series of investigations made with a view to gaining some knowledge of the respiratory activity of the individual wheat grain. Particular attention has been paid to those changes in respiration intensity that are exhibited by grain that is undergoing the process of water absorption and subsequent germination in an adequate supply of air.

¹ Manuscript received December 1, 1941.

Contribution from the Department of Botany, University of Manitoba, Winnipeg, Man. With financial assistance from the National Research Council of Canada.

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Materials and Methods

The experience gained by the author, in the course of researches of a somewhat similar nature upon a variety of germinating seeds (3, 4, 6, 7), showed that the procedure of carrying out experiments in which single individual seeds are used presents certain definite advantages in simplicity over experiments in which the respiration of groups of seeds is measured. In the first place, as different seeds seldom germinate at precisely the same rate, changes in respiration intensity which mark germination stages tend to be obscured when the respiration of a group of seeds germinating together is recorded. Further, where care has to be taken that results are not vitiated by the presence of fungus infection, the single seed experiment makes the detection of such infection and the determination of its effect a relatively simple matter.

The continuous recording of the hourly rates of carbon dioxide output of the seeds under investigation was carried out with a katharometer of a type designed and adapted to this work by the author when collaborating with Stiles (5). The whole apparatus containing the experimental wheat grains was maintained at a constant temperature of $25^{\circ} \pm 0.01^{\circ} \text{C.}$ by immersion in a large thermostatically controlled and continually stirred water-bath.

The procedure adopted throughout the present work was essentially the same as that used in previous researches upon other species. Each wheat grain, after being weighed, was placed in the katharometer respiration chamber along with 0.5 cc. of distilled water with which it was in contact. Carbon dioxide free and moisture saturated air was usually drawn through the chamber which was then sealed. Hourly recording of the amount of carbon dioxide present in the respiration chamber was automatically carried out during the whole period of each experiment. At no time was the concentration of carbon dioxide in the chamber allowed to exceed 0.25%. Respiration rates throughout this paper are expressed as milligrams of carbon dioxide evolved per gram fresh weight of grain per hour.

The wheat used was provided by Dr. Craigie and his colleagues of the Dominion Rust Laboratory, Winnipeg, Man., and was specially tested and selected for freedom from fungus infection. The apparatus used was installed with the aid of grants from the National Research Council of Canada and from the University of Manitoba Research Committee.

The Respiratory Changes During Water Absorption and Germination of Different Varieties of Wheat

The course of respiration during absorption of water and germination in a number of well known varieties of wheat was first determined in order to see if any marked differences in respiratory behaviour existed between them. The varieties examined in this way included both hexaploid (bread) and tetraploid (*durum*) forms, their names being: Thatcher, Marquis, and Renown (hexaploid); Mindum and Iumillo (tetraploid). Fig. 1 shows typical examples of the respiration graphs obtained with single grains of these varieties covering

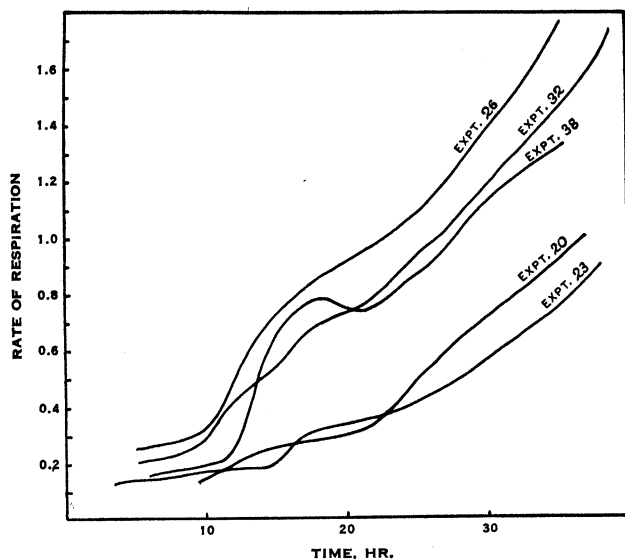


FIG. 1. Course of respiration of single grains of different varieties of wheat during germination. Expt. 26, *Thatcher*; Expt. 32, *Marquis*; Expt. 38, *Renown*; Expt. 20, *Mindum*; Expt. 23, *Iumillo*. Respiration rates are expressed as milligrams of carbon dioxide per gram fresh weight of grain per hour.

the period from shortly after they were placed in contact with water until they were well germinated, with three roots and the coleoptile each several millimetres in length. The numerical data relating to these curves are given in Table I.

TABLE I

RESPIRATORY BEHAVIOUR OF SINGLE GRAINS OF DIFFERENT VARIETIES OF WHEAT DURING GERMINATION

Thatcher, Expt. 26		Marquis, Expt. 32		Renown, Expt. 38		Mindum, Expt. 20		Iumillo, Expt. 23	
Germ'n time, hr.	Resp'n rate	Germ'n time, hr.	Resp'n rate	Germ'n time, hr.	Resp'n rate	Germ'n time, hr.	Resp'n rate	Germ'n time, hr.	Resp'n rate
6.0	0.26	5.5	0.21	5.5	0.15	9.0	0.13	3.5	0.14
9.5	0.30	8.5	0.24	8.5	0.18	13.0	0.22	9.5	0.17
14.0	0.67	11.0	0.37	11.0	0.20	17.0	0.28	14.0	0.18
20.5	0.93	13.0	0.47	13.0	0.38	21.0	0.31	17.0	0.30
23.5	1.02	15.0	0.55	15.0	0.64	25.0	0.51	20.0	0.33
26.5	1.18	17.5	0.68	17.5	0.78	29.0	0.68	26.0	0.45
29.5	1.36	20.5	0.73	20.5	0.73	33.0	0.84	29.0	0.54
32.5	1.55	23.5	0.87	23.5	0.81	36.5	0.99	32.0	0.65
35.0	1.77	26.5	1.00	26.5	0.94	46.5	1.45	35.0	0.76
		29.5	1.15	29.5	1.11			38.0	0.90
		32.5	1.33	32.5	1.19				
		35.5	1.49	35.0	1.32				
		37.5	1.65						

An examination of these data and graphs reveals the fact that the germination process is accompanied by a sequence of three more or less well marked respiratory stages which are common to all of the five varieties of wheat used. The first stage is marked by a slow rise in the respiration rate which begins shortly after the grain is brought into contact with water in the respiration chamber. Following this period of slow rise the respiration rate becomes very definitely accelerated after which it usually again slows off for a time. Later it again increases and follows the more or less uniformly rising course which is associated with the more advanced stages of germination.

From a further examination of the graphs given in Fig. 1 it seems clear that on grounds of respiratory behaviour the bread wheats form a group distinct from the *durum* wheats. In the bread wheats (Renown, Marquis, and Thatcher) the initial stage with its slowly rising respiration rate usually extends over a period of about 10 hr. although occasional examples were encountered in which this period was considerably longer or considerably shorter than the average. On the other hand, in the *durum* wheats (Mindum and Iumillo) this initial stage was usually of longer duration. Further, as is clearly indicated in Figs. 1, 2, and 3, the four respiratory stages already described are much more definitely pronounced in the graphs of the bread wheats than in those of the *durum* wheats. An additional fact that is illustrated in Figs. 2 and 3, is that, within each of the two groups (*durum* wheats and bread wheats) different varieties do not appear to differ from each other in respiratory behaviour more than do individual grains of a single variety. In this connection, Fig. 2 gives graphs of three individual grains of Renown wheat, while Fig. 3 gives similar graphs of two individuals of Iumillo and two of Mindum.

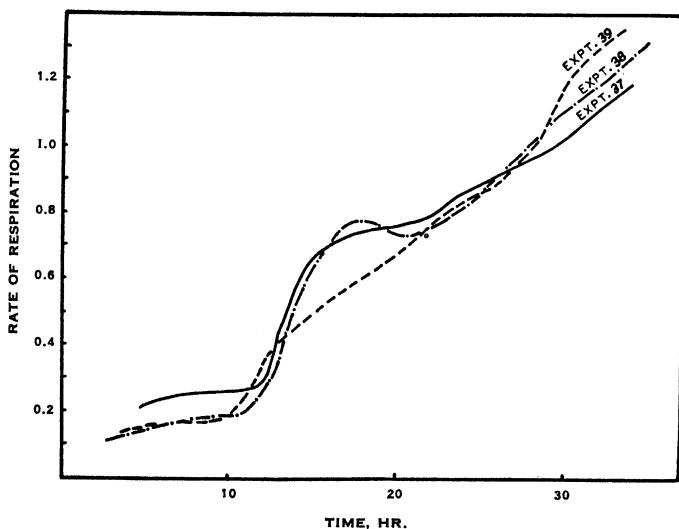


FIG. 2. Course of respiration of three individual grains of Renown wheat.

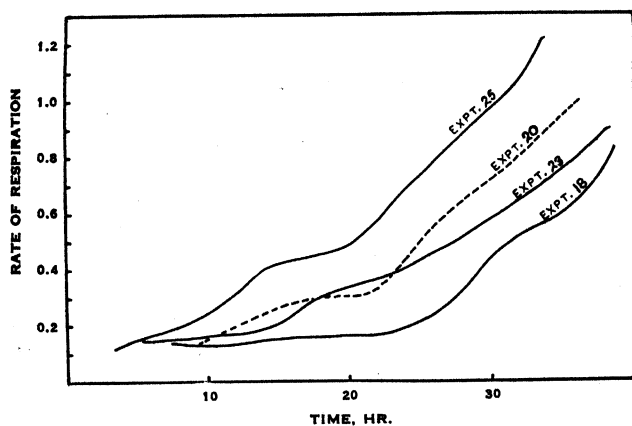


FIG. 3. Course of respiration of four individual grains of durum wheat. Expt. 25 and 23, Iumillo; Expt. 20 and 18, Mindum.

The Relationship Between the Rate of Water Absorption and the Course of Respiration

The rate of water absorption by samples of *durum* and bread wheats under the experimental conditions used and during the period when the changes in respiration rate just described take place are given in Fig. 4. The varieties used were Mindum and Renown and their initial moisture contents were 11.40 and 12.10%, respectively. It will be observed that the two varieties do not show sufficient difference in their rates of water absorption to be significant in causing their observed differences in respiratory behaviour. Further, these curves show that during the first two hours of the experiment, the water content of the grains reaches a value exceeding 20%, while by the end of the first 10-hr. period the grains had attained a water content of between 30 and 40%. The slowness of the initial rise in respiration rate already men-

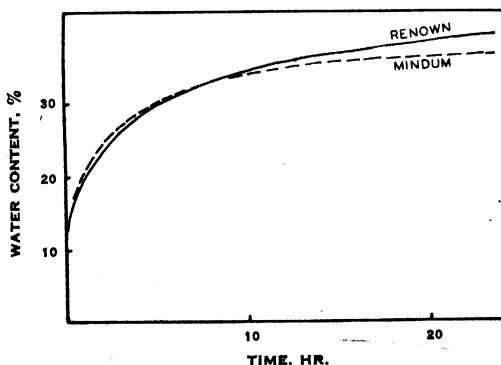


FIG. 4. Graphs showing rates of water absorption of Renown and Mindum wheats.

tioned would not therefore appear to be the result of shortage of water in the respiring tissues.

With a view to possibly throwing further light upon this question, a number of experiments were carried out in which the wheat grains were soaked for a number of hours in water maintained at a temperature of approximately 0° C. immediately before introducing them into the respiration apparatus at the experimental temperature of 25° C. The data and graphs given in Table II and Fig. 5 show the results of this treatment.

TABLE II

RESPIRATORY BEHAVIOUR OF SINGLE GRAINS OF RENOWN WHEAT DURING GERMINATION SUBSEQUENT TO SOAKING IN WATER AT 0° C.

Expt. 53		Expt. 54		Expt. 57	
Water content at beginning of experiment = 26.1%		Water content at beginning of experiment = 36.0%		Water content at beginning of experiment = 27.0%	
Germination time, hr.	Respiration rate*	Germination time, hr.	Respiration rate*	Germination time, hr.	Respiration rate*
3.0	0.11	3.0	0.20	2.5	0.11
6.0	0.16	5.5	0.22	5.5	0.12
9.0	0.18	8.5	0.26	8.5	0.14
12.0	0.28	11.5	0.34	11.5	0.10
15.0	0.38	14.5	0.38	14.5	0.12
18.0	0.48	17.5	0.42	17.5	0.29
21.0	0.57	20.5	0.52	20.5	0.39
24.0	0.67	23.5	0.59	23.5	0.38
27.0	0.72	26.5	0.64	29.5	0.46
30.0	0.80	29.5	0.67	32.5	0.54
33.0	0.86	32.5	0.72	35.5	0.62
		35.5	0.78		

* Respiration rates are expressed as milligrams of carbon dioxide per gram fresh weight of grain per hour.

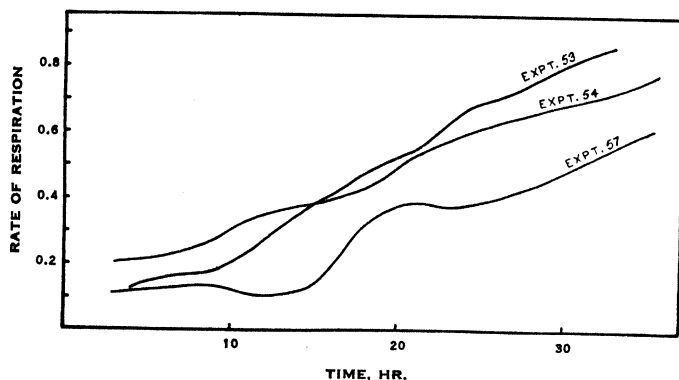


FIG. 5. Course of respiration of single grains of Renown wheat after soaking in water at 0° C.

It will be seen that this previous soaking did not bring about any acceleration of the rate at which respiration increases during the initial germination stage. The slow respiration increase must therefore be due, under the experimental conditions, to some factor other than deficiency of water.

The Effect of Fungus Infection

During the course of these experiments a number of grains developed growths of fungus hyphae while in the plant chamber. In all such grains germination was inhibited while the respiration rate either remained low from the start or began to rise in the normal way only to fall off again after between 10 and 20 hr. from the beginning of the experiment. The respiration curves for three typical examples of infected grains are given in Fig. 6.

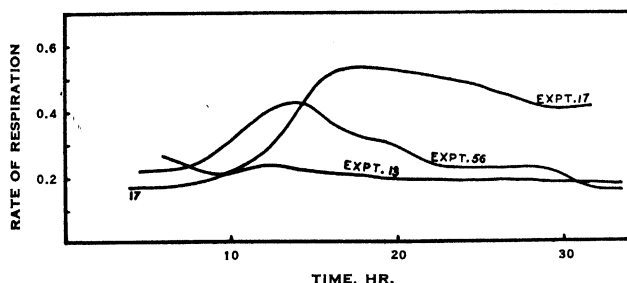


FIG. 6. Course of respiration of fungus infected wheat. Expt. 17, Mindum; Expt. 56, Renown; Expt. 13, Thatcher.

Discussion of Results

A remarkable fact that is revealed by the foregoing data is the extraordinarily high rate of acceleration that is exhibited by the carbon dioxide output of the wheat grain immediately it is brought into contact with water. Taking a value obtained from the data of Bailey and Gurjar (1) namely: 0.0002 mg. carbon dioxide per gram dry weight of grain per hour for the respiration of wheat with 12.5% moisture content and at a temperature of 37.8° C. as some indication of the carbon dioxide output of normal air-dry grain, it would appear that the respiration rate increases by something of the order of 1000 times during the first five hours from the time the grain is brought into contact with water. By this time the water content of the grain has reached about 30%. While taking Bailey and Gurjar's figures as being approximately correct for wheat under storage conditions at the exceptionally high experimental temperature used, it is intended to carry out further research into this question at a temperature nearer to the optimum for germination and under more definitely controlled conditions regarding oxygen supply to the grain.

It seems definite that there exists in dry wheat a fully developed oxidizing system and an immediately available supply of respiratory substrate. In those experiments in which the measurement of the respiration rate was begun immediately after the grain was placed in contact with water, the reaction

was at the start only limited by the amount of water present in the grain. In those experiments in which the grain was previously soaked in water maintained near freezing point, temperature was obviously the limiting factor. A tentative visualization of the sequence of events that result in the three already described respiratory stages may now be suggested.

The first stage recorded in these experiments, namely, that in which the respiration intensity is rising rather slowly and at a diminishing rate, should more accurately be looked upon as an approach towards the conclusion of the first stage. Its beginning must actually be marked by a very rapid rise in respiratory activity, which, owing to experimental limitations, was not recorded. This rise closely follows the initial rapid intake of water by the grain in the experiments where respiration was measured during the water absorption process, while in the experiments with grain previously soaked in water maintained at freezing point, the rapid rise closely followed the rise in temperature from 0° to 25° C. The recorded slowing down of this initial increase which must now be considered as the end of the first respiratory stage can be explained as the natural course followed where the substrate is limited in amount and where the continuation of the reaction depends upon diffusion for the maintenance of contact between the oxidizing system and the substrate.

The second respiration stage with its initial increase followed by decrease in rate of acceleration would appear to mark the mobilization of new substrate resulting from the hydrolysis of reserves present in the endosperm and the subsequent saturation of the immediately available oxidation system.

The final stage with its maintained rate of acceleration of respiration can readily be understood as the outcome of the development of new and extending centres of respiratory activity in the form of meristems in the rapidly growing seedling.

With regard to the effect of fungus infection on the course of respiration, it is obvious from the results described that the presence of fungus reduced rather than increased the carbon dioxide output of the grain under the experimental conditions used in this work. A significant feature of all three of the experiments recorded in Fig. 6 was the fact that while at the close of the experiments the wheat grains were enveloped in a considerable growth of mycelium, the respiration rate was still falling. This would seem to suggest that the carbon dioxide output of the fungus may not be so great as Larmour's (2) experiments seem to indicate. It is evident, however, that the question of the effect of fungus infection upon the respiration of germinating seeds needs further investigation.

In conclusion it should be pointed out that the experiments described in this paper deal with the respiratory activity of wheat germinating under conditions approaching the optimum in regard to water supply, aeration, and temperature. The results are therefore in no way comparable with those obtained by other workers with grain maintained under bulk storage conditions

where in many cases carbon dioxide is allowed to accumulate round the grain and where the grain may be subjected to temperatures that are definitely injurious, if not fatal, to living tissues. On the other hand, the present experiments indicate the possible nature of some of the physiological processes that become increasingly active in a wheat grain when external conditions are changed from those normally present during storage to others that are favourable to germination.

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INHIBITION OF MICRO-ORGANISMS BY A TOXIC SUBSTANCE PRODUCED BY AN AEROBIC SPORE-FORMING BACILLUS¹

BY H. KATZNELSON²

Abstract

A thermostable diffusible substance produced by an aerobic spore-forming bacillus in a potato dextrose peptone medium containing 2% dextrose, 1% peptone, and adjusted to pH 6.5 was found to inhibit the growth of 77 out of 81 species of parasitic and saprophytic fungi. Actinomycetes were more tolerant than fungi, though some were completely inhibited. The majority of streptococci, staphylococci, bacilli, lactobacilli, and clostridia tested were suppressed by the toxic medium. Gram negative organisms were unaffected. *Bacillus subtilis* and, to some extent, *B. cereus* and *B. pumilus* produced thermostable substances toxic to *Rhizoctonia Solani*. Soil, bentonite, and activated charcoal completely adsorbed the toxic agent, agar was less effective and talc not at all. The toxic substance passed through cellophane, parchment, and collodion, resisted autoclaving for 30 to 45 min. at 15 lb., but was destroyed rapidly by heating in alkaline, but less rapidly in acid solutions; it was not inactivated by aeration and retained its potency for many months at 0° C.; ether, chloroform, benzene, ethyl acetate, and *N* butyl alcohol could not remove it from the toxic medium but it could be eluted partially from charcoal with 95% ethyl alcohol. The substance has not as yet been identified.

Introduction

The subject of antagonism and association among micro-organisms has been thoroughly reviewed recently (3, 5, 6, 7, 8) and need not be discussed further. It will suffice to call attention to the keen interest displayed in recent years in the question of antagonism among micro-organisms owing to the possibility of using that principle in controlling certain pathogenic or otherwise undesirable organisms. The recent work of Dubos *et al.* and others (7) gives some indication of the ultimate value of such studies in medicine. The investigations of various plant pathologists also point to a growing appreciation of the possible importance of antibiotic reactions in the control of various plant pathogenic organisms under natural conditions (7).

Various members of the genus *Bacillus* have been found to produce substances toxic to other organisms (7). This paper is a brief report of the results of some studies on the antagonistic action of another member of this genus towards fungi, bacteria, and actinomycetes (4); the bacillus studied (*Bacillus* sp.) was first shown to produce a thermostable toxin active against the fungus *Rhizoctonia Solani* by Cordon and Haenseler (1).

¹ Manuscript received January 2, 1942.

Contribution from the Department of Plant Pathology, Rutgers University, New Brunswick, N.J., the Department of Bacteriology, Cornell University, Ithaca, N.Y., and the Division of Bacteriology and Dairy Research, Department of Agriculture, Ottawa, Canada. (Journal Series No. 141.)

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Experimental

The toxic material was produced by growing the bacillus in shallow layers of potato dextrose medium (2) containing 1% peptone. After seven days incubation at 28° C. the pellicle produced by the organism was removed and the comparatively clear toxic culture solution, henceforth designated the toxic medium, was utilized for various tests. When a clear toxic medium was desired the culture solution was centrifuged.

INHIBITORY ACTION OF TOXIC MEDIUM ON FUNGI, BACTERIA, AND ACTINOMYCETES

The toxic medium was diluted with potato dextrose peptone broth adjusted to pH 6.5, sterilized, and inoculated with 81 species of saprophytic and phytopathogenic fungi representing 50 genera and including representatives of the four major fungal groups. With the exception of four species that grew poorly, none of the fungi grew in the undiluted toxic medium, but all grew in the fresh substrate.

A number of species of actinomycetes were similarly tested. These organisms appeared to be more tolerant than fungi, as only three out of eight were completely inhibited by the undiluted toxic medium; three were partially suppressed and two, not at all.

Representative aerobic Gram positive and Gram negative bacteria were next studied. The medium most suitable for each particular organism was diluted with clear toxic medium (pH 6.5), sterilized, inoculated, and incubated at a suitable temperature. Of 21 streptococcus cultures (including representations of the major groups of genus *Streptococcus*) 17 were inhibited. All six cultures of staphylococci were inhibited as were six of seven species of spore-forming bacilli. *Lactobacillus acidophilus* and *L. casei* strains were quite tolerant, but three of five strains of *L. bulgaricus* were completely suppressed. Gram negative organisms such as *Escherichia coli*, *Proteus vulgaris*, *Aerobacter aerogenes*, *Pseudomonas fluorescens*, etc., were not appreciably affected.

Twenty pathogenic anaerobes of the genus *Clostridium*¹ (representing 12 species) were found to be susceptible to the toxic medium in various degrees. Of these, 11 were completely inhibited by all the dilutions of toxic medium tested, five were suppressed only by higher concentrations and four grew, though poorly, in the undiluted toxic substrate.

All the organisms that were inhibited by the toxic medium grew well when inoculated into this medium after it had been treated with activated charcoal, filtered, adjusted to the necessary pH, and sterilized.

In Table I are presented data concerning the influence of different quantities of toxic medium on test organisms selected at random from the groups mentioned above. Fungi were on the whole less tolerant to low concentrations

¹ The author is indebted to Mr. Louis Greenberg, Laboratory of Hygiene, Department of Pensions and National Health, Ottawa, Canada, for the cultures of clostridia and to Dr. N. R. Smith of the United States Department of Agriculture, Washington, D.C., for the cultures of bacilli.

TABLE I

INFLUENCE OF DIFFERENT CONCENTRATIONS OF TOXIC MEDIUM ON THE GROWTH OF VARIOUS ORGANISMS

Organism tested	Concentration of toxic medium (% by volume)							Organism tested	Concentration of toxic medium (% by volume)						
	0	10	20	40	60	80	100		0	10	20	40	60	80	100
<i>Aphanomyces</i> sp.	4	2	0	0	0	0	0	<i>Bacillus</i>							
<i>Aspergillus</i> sp.	4	2	2	2	0	0	0	<i>mycoides</i>	4	0	0	0	0	0	0
<i>Aspergillus oryzae</i>	4	3	2	2	2	2	2	<i>brevis</i>	4	0	0	0	0	0	0
<i>Botrytis alvei</i>	4	0	0	0	0	0	0	<i>subtilis</i>	4	4	4	4	4	3	0
<i>Cephalothecium roseum</i>	4	0	0	0	0	0	0	<i>Clostridium</i>							
<i>Chaetomium</i> sp.	4	0	0	0	0	0	0	<i>botulinum</i>	4	4	3	0	0	0	0
<i>Citromyces</i> sp.	4	3	3	2	2	2	2	<i>chauvei</i>	4	4	4	4	4	3	2
<i>Colleotrichum phomoides</i>	4	0	0	0	0	0	0	<i>norvi</i>	4	0	0	0	0	0	0
<i>Fusarium culmorum</i>	4	1	1	0	0	0	0	<i>tetani</i>	3	0	0	0	0	0	0
<i>Gladiadium</i> sp.	4	1	0	0	0	0	0	<i>welchii</i>	4	0	0	0	0	0	0
<i>Gloeosporium</i> sp.	4	0	0	0	0	0	0	<i>Lactobacillus</i>							
<i>Helminthosporium sativum</i>	4	0	0	0	0	0	0	<i>acidophilus</i>	4	4	4	4	4	4	1
<i>Humicola</i> sp.	4	0	0	0	0	0	0	<i>bulgaricus</i>	4	0	0	0	0	0	0
<i>Monoascus purpureus</i>	4	0	0	0	0	0	0	<i>Leuconostoc citrovorus</i>	4	3	2	0	0	0	0
<i>Monilia sitophila</i>	4	0	0	0	0	0	0	<i>Staphylococcus</i>							
<i>Mycogone</i> sp.	4	2	1	0	0	0	0	<i>aureus</i>	4	4	4	0	0	0	0
<i>Paeclomyces</i> sp.	4	0	0	0	0	0	0	<i>cereus</i>	4	0	0	0	0	0	0
<i>Penicillium</i> sp.	4	0	0	0	0	0	0	<i>Streptococcus</i>							
<i>Penicillium digitatum</i>	4	3	3	2	2	2	2	<i>pyogenes</i>	4	2	1	0	0	0	0
<i>Phytophthora infestans</i>	4	3	2	1	0	0	0	<i>mastitidis</i>	4	4	4	4	2	0	0
<i>Phymatotrichum omnivorum</i>	4	3	2	0	0	0	0	"animal pyogenes"	4	0	0	0	0	0	0
<i>Rhizopus</i> sp.	4	0	0	0	0	0	0	"human pyogenes"	4	4	4	4	2	0	0
<i>Rhizoctonia Solani</i>	4	0	0	0	0	0	0	<i>liquefaciens</i>	4	4	4	4	4	4	3
<i>Sclerotinia minor</i>	4	2	2	0	0	0	0	sp. (Group K)	4	0	0	0	0	0	0
<i>Thielaviopsis</i> sp.	4	2	2	0	0	0	0	<i>lactis</i>	4	4	4	4	4	4	3
<i>Torula</i> sp.	4	1	0	0	0	0	0	<i>salivarivus</i>	4	4	3	3	2	0	0
<i>Trichoderma</i> sp.	4	4	4	1	0	0	0	<i>Escherichia coli</i>	4	4	4	4	4	4	2
<i>Actinomyces</i>								<i>Pseudomonas fluorescens</i>	4	4	4	4	4	4	4
<i>asteroides</i>	4	4	4	4	2	0	0								
<i>cellulosae</i>	4	4	3	0	0	0	0								
<i>griseus</i>	4	4	4	4	4	4	4								
<i>scabies</i>	4	4	3	2	2	2	2								

NOTE: 4 = good growth; 3 = medium; 2 = fair; 1 = slight; 0 = no growth.

of the inhibitory material than actinomycetes and bacteria. Considerable variation in susceptibility was obtained with different species in one genus.

PRODUCTION OF SUBSTANCES TOXIC TO *Rhizoctonia Solani* BY VARIOUS SPECIES OF GENUS *Bacillus*¹

The bacilli listed in Table II were inoculated into 100 ml. of potato dextrose peptone broth at pH 6.8. All flasks were incubated at 30° C. for seven days after which the cultures were diluted with the potato broth and sterilized. *Rhizoctonia Solani* was then inoculated into every flask.

B. subtilis evidently produces a thermostable substance which is almost as toxic to the fungus as that of the bacillus under investigation. *B. cereus* and to some extent *B. pumilus* also liberate some heat resistant inhibitory factors. These substances were also adsorbed by charcoal.

¹ See Footnote, p. 170.

TABLE II
PRODUCTION BY DIFFERENT BACILLI OF SUBSTANCES TOXIC TO *Rhizoctonia Solani*

Organism tested	Concentration of toxic culture medium (% by volume)							
	1	3	5	10	25	50	75	100
<i>B. brevis</i>	4	4	4	4	4	4	4	4
<i>B. cereus</i>	4	3	2	1	1	0	0	0
<i>B. mycoides</i>	4	4	4	4	4	4	2	2
<i>B. megatherium</i>	4	4	4	4	4	3	2	1
<i>B. niger</i>	4	4	4	4	4	2	2	1
<i>B. pumilus</i>	4	4	4	4	4	3	1	0
<i>B. subtilis</i>	3	2	0	0	0	0	0	0
<i>Bacillus</i> sp. used in all previous experiments	2	0	0	0	0	0	0	0

NOTE: 4 = good growth of *Rhizoctonia* (as in fresh medium); 3 = medium; 2 = fair; 1 = slight; 0 = no growth.

CONCENTRATION OF ACTIVE SUBSTANCE

In view of the low concentration of the inhibitory agent produced by the bacillus under investigation in the toxic medium, an attempt was made to stimulate production of the principle by growing the organism in different media at various pH levels. Inorganic substrates containing organic and inorganic forms of nitrogen (with and without dextrose), nutrient and infusion broths, and potato extract with varying concentrations of dextrose, sucrose, and peptone were all inoculated with the bacillus and the cultures tested against *Rhizoctonia*. Maximum production of toxic material was obtained with potato extract plus 2% dextrose and 1% peptone. Smaller amounts of both dextrose and peptone resulted in lower yields of the toxic agent. Sucrose was only slightly less effective than dextrose. The bacillus grew well and produced the toxic substance best between the limits of pH 5.5 and 6.8. Agitation of the growing culture was unfavourable for production of the inhibitory principle.

Various attempts to adsorb the toxic agent showed that activated carbon, bentonite, and soil removed it rapidly and completely; agar was less effective and talc, not at all. The toxic substance could be removed from charcoal with 95% alcohol (hot or cold) although considerable loss occurred. Removal of the alcohol by distillation resulted in the production of a water and acid insoluble, alkali soluble precipitate from which some of the toxic material could be removed by washing with distilled water and the substance could be concentrated by boiling off the excess water. The nature of this toxic substance is not known.

Acetone, buffer solutions, and methyl alcohol were less effective in removing the toxic agent from charcoal than 95% alcohol. The principle could not be removed from toxic medium with ether, chloroform, benzene, ethyl acetate, or *N* butyl alcohol, nor could it be precipitated with acid, 95% ethyl alcohol, or ammonium sulphate. It passed readily through collodion, cellophane, and parchment membranes at a neutral or slightly acid reaction. The toxic material resisted autoclaving for as long as 30 to 45 min. at 15 lb. pressure;

it was destroyed quite rapidly by heating in alkaline but was more tolerant to heating in acid solutions. The toxic medium retained its inhibitory character after five-months storage in sealed containers at 0° C. but was less stable at 50° C. It was not destroyed rapidly when kept at room temperature in cotton-plugged flasks. Aeration of toxic medium for seven days did not inactivate it.

Discussion

The production by certain organisms of substances toxic to others is by no means new, although it is rare that such toxic principles are inhibitory to so large a number of organisms, saprophytes and parasites alike, as were studied in the above experiments. The consistent inhibition of fungi by toxic substances produced by certain bacteria is particularly interesting, and may be an important factor in altering the fungus flora of the soil and thus in affecting the distribution of certain soil phytopathological organisms.

Aside from the academic considerations of these antagonistic reactions between the bacillus and the variety of organisms tested, there are practical implications that may find important application in different fields. It was noted, for example, that most of the staphylococci, streptococci, and clostridia (the majority of which are pathogenic to animals) were inhibited by the toxic principle. Many of the fungi studied are serious plant pathogens whose eradication would be of inestimable benefit; these were also repressed by the toxic agent. The extensive and effective utilization of such a substance, however, depends on a number of important factors: concentration of the material, its activity under a wide variety of conditions both in the laboratory and in the field, the ease of its production in quantity by the organism and of its chemical synthesis should its structure be discovered, and its effect on the host plant or animal to be treated. It is clear, therefore, that the problems attending the possible utilization of this and other antibiotic substances of biological origin in general, for the control of pathogenic organisms, are many and complex, and require much further investigation.

Acknowledgment

The author is indebted to Dr. C. M. Haenseler, Department of Plant Pathology, New Jersey Agricultural Experiment Station, New Brunswick, N.J., for his many helpful suggestions in the course of the work and in the preparation of the manuscript.

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STUDIES ON THE GROWTH IN SOIL AND THE PARASITIC ACTION OF CERTAIN *RHIZOCTONIA SOLANI* ISOLATES FROM WHEAT¹

BY I. D. BLAIR²

Abstract

An adaptation of the Rossi and Chododny glass slide technique was found to be an effective means of measuring the growth of *Rhizoctonia Solani* in soil. After a 6 day and a 12 day period, the extent of growth of 11 isolates of this fungus was, for each growth period, less in a vertical than in a radial direction. Certain isolates grew faster than others. A comparison of the radial growth of a faster and of a slower growing isolate at soil depth of 2, 4, and 6 in. showed that the extent of growth decreased with depth, being significantly greater for both isolates at the 2 in. than at the 6 in. level.

In pathogenicity tests on wheat with 10 of these isolates, the disease rating for each isolate was greater in natural than in steam sterilized soil, and in soil with a proportion of inoculum to soil of one to six than of one to three. The addition of cellulosic organic material, grass- or straw-meal, to unsterilized soil was effective in reducing the parasitic action of all isolates. Two distinct types of injury were observed: the one, a severe form of root injury, resulting in reduced plant growth; the other, a girdling of the coleoptile or lower stem tissue, usually unaccompanied by adverse effects on plant growth. The first type was produced by two slow growing isolates of English origin, the second by faster growing isolates of Canadian origin. On the basis of these differences, it is suggested that the root injuring isolates be regarded as a variety of *R. Solani* Kühn.

Introduction

Species of *Rhizoctonia* have frequently been isolated from wheat crowns and roots (1, 9, 13, 17, 18, 19), although it was not until 1928 that Samuel (11), in South Australia, described an actual *Rhizoctonia* disease of wheat. The same disease also occurred on oats. Subsequently the nature of this disease, caused by *Rhizoctonia Solani* Kühn, was fully studied by Samuel and Garrett (12) and Hynes (6, 7, 8). According to these Australian workers, this disease usually occurs in patches and is characterized by spindly, stunted plants with stiff, inwardly rolled leaves. It was found that the greater part of the length of affected roots was destroyed, leaving only short brown stumps, a little more than 1 cm. long. In areas where soil infestation appeared light, some of the plants appeared able to throw off the attack, forming a new root system, but affected plants exhibited delayed maturity owing to the initial check given by the seedling attack.

In view of the Australian evidence that a definite *Rhizoctonia* disease of wheat does occur, the present investigation has been devoted (1) to an examination of some of the growth characteristics in the soil of isolates of *R. Solani* from wheat plants, and (2) to studies of certain aspects of their parasitism on the wheat plant.

¹ Manuscript received October 3, 1941.

Contribution No. 680 from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada.

In this paper are embodied some of the results of an investigation conducted by the author at the Dominion Plant Pathological Laboratory, Winnipeg, Man., during the tenure of the Macmillan Brown Agricultural Research Scholarship of the University of New Zealand, Wellington, N.Z.

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I. Growth Studies of Isolates in Soil

MATERIALS AND METHODS

Three different experiments were conducted to determine the extent and nature of growth in soil of *R. Solani* isolates. The isolates were obtained from wheat crowns, roots, and seed, of English and Canadian origin, and were cultured initially on potato dextrose agar. The soil substrate consisted of Red River (Manitoba) surface soil, a heavy clay loam, which was air-dried and sieved through a 2 mm. mesh and then mixed with dry sand in the proportion of five parts of soil to one of sand. The levels of moisture are indicated later in connection with the different experiments.

The extent of growth of *R. Solani* isolates in the soil was determined by an adaptation of the Rossi (10) and Cholodny (3) glass slide technique. Glass slides, cleaned in acidulated potassium bichromate, were placed in position, as later described, in the soil within the pots or jars used as containers. The isolate under study was introduced in the form of a 6 mm. agar disk against each slide at a marked point, the disks being cut from the peripheral mycelial growth on the cultural plates. The soil in each container was then covered to a depth of $\frac{1}{2}$ in. with a layer of sand adjusted to the same moisture level as the soil which it covered. The containers were randomized on a table in the laboratory and maintained at room temperatures. After inoculation, the soil was maintained at the previously adjusted degrees of saturation by daily weighings with additions of water where necessary. At the end of 6 or 12 days after inoculation, the slides were removed from the containers, detached from the soil, and fixed and stained in a steaming solution of 5% erythrosin in 5% carbolic acid.

During the interval allowed for growth, the fungus grew out through the soil from the points of inoculation. Mycelial strands came in contact with the slides. By means of a high or low power microscope objective, the course of the fungus along the slide could easily be followed and measured from the original mark of inoculation to the terminal extremity of growth. Regardless of the density of growth of the other micro-organisms adhering to the slides when taken from the soil, it was possible to trace accurately on the slides the extent of growth of the inoculated fungus from the focal points. In view of the striking morphological characteristics of the mycelium (4), it seemed that *R. Solani* was eminently suited for a study using such a technique. It is doubtful, however, if the method could be applied to other fungi less readily distinguishable in a mixed soil microflora.

EXPERIMENTAL RESULTS

Vertical and Radial Growth

In order to compare the ability of different isolates to grow through the soil, 11 isolates of *R. Solani* were studied in respect to their extent of vertical and radial growth. Two experiments were carried out in which the water content of the soil and of the sand mulch were adjusted to 40% of saturation

by hand mixing of the soil and water. In the first, the slides were arranged vertically in the soil and an inoculum disk was placed against each slide at a marked point one centimetre below the soil surface. For each isolate there were six 4 in. clay pots with four vertically arranged slides. The slides were removed from three pots at the end of six days, and from the other three at the end of 12 days. Twelve slides were therefore available for measurement at each of the two sampling intervals. In the second experiment, the glass slides were placed on edge, radially, in glazed clay crocks. The disk of inoculum was placed at the edge of each slide in contact with the wall of the containers at a point one centimetre below the soil surface. For each isolate, there were four crocks with six slides, again permitting measurements from 12 slides at the 6 and the 12 day sampling periods. In the first experiment, the temperature ranged from 17.2° to 23.8° C., with a mean of 19.1° C.; and, in the second, from 18.0° to 23.8° C., with a mean of 19.4° C. The data for these two experiments are given in Table I.

TABLE I

THE EXTENT OF GROWTH OF 11 ISOLATES OF *Rhizoctonia Solani* FROM WHEAT IN SOIL AT 40% OF SATURATION

Slides vertically arranged			Slides radially arranged		
<i>R. Solani</i> isolate	Growth, cm.		<i>R. Solani</i> isolate	Growth, cm.	
	After 6 days	After 12 days		After 6 days	After 12 days
<i>S.</i> 21	2.7	3.5	<i>S.</i> 21	3.4	5.1
<i>S.</i> 22	2.7	3.7	<i>S.</i> 25	3.2	5.2
<i>M.</i> 2	2.6	3.1	<i>B.</i> 140	3.2	4.8
<i>S.</i> 25	2.5	3.7	<i>S.</i> 22	3.1	5.3
<i>B.</i> 140	2.5	3.2	<i>S.</i> 23	3.1	4.8
<i>M.</i> 3	2.5	3.0	<i>M.</i> 2	2.9	4.4
<i>M.</i> 4	2.4	3.0	<i>M.</i> 4	2.8	4.2
<i>S.</i> 23	2.3	3.2	<i>M.</i> 3	2.7	4.2
<i>B.</i> 5-3	1.9	2.4	<i>B.</i> 5-3	2.7	3.5
<i>R.</i> 6	1.7	2.1	<i>R.</i> 5	2.1	3.3
<i>R.</i> 5	1.6	2.0	<i>R.</i> 6	2.0	3.3
Necessary difference, 5% level	0.54	0.56	Necessary difference, 5% level	0.62	0.56

The data in Table I show that the extent of growth of all the isolates was greater in the lateral plane of the soil than in the vertical plane. They show also that certain isolates (e.g., *S.* 21 and *S.* 22) grew significantly faster than certain other isolates (*R.* 5 and *R.* 6), whether in the lateral or vertical direction, or for the shorter or longer period of growth. Two or three of the isolates (*M.* 2, *M.* 3, and *M.* 4) occupy a more or less intermediate position in respect to their rate of growth in the soil. It would appear, therefore, that, although the organism may grow centrifugally from a focal point in the soil, it is less inclined to grow downward than laterally, no matter what its growth rate

may be. From Table I, it can readily be calculated that, for the respective isolates, the difference in the amount of growth for the 6 day period and the 12 day period was from two to three times as great in the lateral direction as in the vertical direction. This fact would indicate a definite retardation of vertical growth as the isolates reached the lower levels in the soil, an aspect of the problem examined in the third experiment.

Growth at Different Soil Depths

A comparison was made of the extent of growth of *R. Solani* at different soil depths. The same Red River clay loam was mixed with sieved dry sand in the following proportions: (1) soil five parts, sand one part; (2) soil three parts, sand two parts; and (3) soil one part, sand one part. Water was added to the mixtures to provide two levels of saturation, namely, 33% and 50%. The containers were glazed clay crocks. Sufficient soil was added to each crock to reach a level six inches from the top. Six glass slides were arranged on edge in a radial manner and inoculations were made against each at a marked point one centimetre below the soil surface. More soil was added to reach a level four inches from the top. Here the same procedure with slides and inoculum was followed, and a further addition of soil brought the soil surface to within two inches of the top, where another set of slides with inoculum was placed in position. Each crock was finally filled to within half an inch of the top, and a half-inch sand mulch, adjusted to either 33% or 50% of saturation capacity, provided the surface cover.

A typical fast growing isolate, *S. 22*, and a slower growing one, *B. 5-3*, served as inoculum. The experiment was made in duplicate, thus providing 12 slides for each variable. The crocks were weighed daily, but at room temperature, water losses were slight. After 12 days, the slides were carefully removed for fixing and staining. Complete data for the effect of different soil depths, soil mixtures, and soil moistures on the growth of the two isolates, *S. 22* and *B. 5-3*, are given in Fig. 1. It is evident from this figure that, in all soil mixtures used, both isolates grew better at a soil depth of 2 in. than at a soil depth of 4 in., and at 4 in. than at 6 in. of depth. In all instances, growth was significantly greater at a depth of 2 in. than of 6 in. Generally, both isolates made the most rapid growth in a soil mixture consisting of three parts of black clay loam and two parts of sand. Although the faster growing isolate *S. 22* grew better in soil with a moisture content of 33% of saturation than in soil with a 50% of saturation, the growth rate of the slower growing isolate *B. 5-3* was much the same at both moisture levels.

The results of the analysis of variance for the data are given in Table II. From this table, it is evident that statistically significant effects are shown for both isolates for soil depths and soil mixtures. Although isolate *S. 22* shows highly significant moisture effects, there are no statistically significant differences in the growth of isolate *B. 5-3* attributable to varying soil moisture contents.

From the results presented in Fig. 1 and Table II, it seems clear that the growth of *R. Solani* is markedly influenced by conditions existing at the depth level in the soil at which the fungus may be growing. The upper soil stratum provides environmental conditions more favourable for the growth of the fungus than do the lower soil levels. It would seem that the differences in

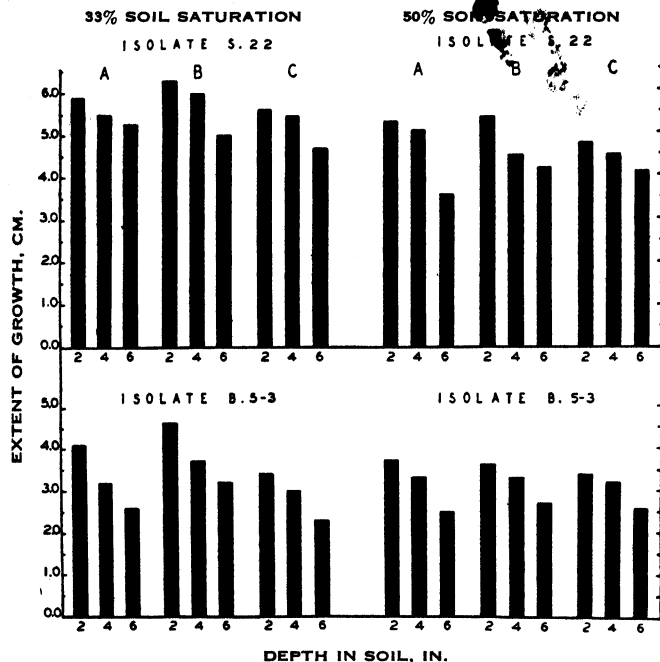


FIG. 1. Bar diagram of data relative to the growth of two isolates of *R. Solani* at three levels of depth in soil. Growth measurements (cm.) 12 days after inoculation. A = soil 5, sand 1; B = soil 3, sand 2; C = soil 1, sand 1.

TABLE II

ANALYSIS OF VARIANCE OF DATA ON THE GROWTH OF TWO ISOLATES OF *R. Solani* AT THREE DIFFERENT SOIL DEPTHS

Source of variance	D.f.	Mean square	
		Isolate S. 22	Isolate B. 5-3
Depths	2	21.055**	23.855**
Soil mixtures	2	2.525*	4.985**
Moistures	1	39.000**	2.320
Depths × soil mixtures	4	0.395	0.255
Depths × moistures	2	0.320	0.785
Soils × moistures	2	0.410	1.565
Depths × soils × moistures	4	2.245**	1.940*
Error	198	0.592	0.709

* Exceeds mean square error, 5% level of significance.

** Exceeds mean square error, 1% level of significance.

growth at the three levels examined are closely associated with soil moisture and soil aeration. In crock experiments, the soil moisture content increases with increasing depth, and, in consequence, soil aeration would be better at the 2 in. level than at the 6 in. level. As the growth of *R. Solani* was significantly greater at the former than at the latter level, it would seem that low moisture content and good aeration favour the growth of this fungus in the soil. These conclusions lend support to the belief that if one wishes to measure the maximum growth of *R. Solani* in the soil, such measurements should be made in the upper part of the soil column, and, in the event of slides being used as the implement of growth measurement, they should be arranged in a horizontal plane.

II. Pathogenicity Studies of Isolates on Wheat

MATERIALS AND METHODS

Experiments were conducted to determine the pathogenic capabilities of the *R. Solani* isolates on wheat. The soil was composed of five parts of Red River surface clay loam and one part of sand. Soil treatments will be described in connection with the different experiments. The culture medium was prepared in Erlenmeyer flasks by mixing 2% finely ground corn-meal in washed sand adjusted to 30% of its saturation capacity. After autoclaving, each flask of medium was inoculated with six mycelial disks of the isolate to be tested, taken from the periphery of growth on a potato dextrose agar plate. The isolates were allowed to develop in the medium for 18 days before soil inoculation. Mixing of the inoculum with the soil was done by shaking in a sterile glass container one part of inoculum to three parts of soil, and one part of inoculum to six parts of soil. Immediately, thereafter, the water content of the mixtures was adjusted to 40% of saturation. Each mixture was then placed in 4-in. clay pots, and, after an interval of three days, seed of Regent wheat, hand selected and surface sterilized, was sown. After emergence, all seedlings over 12 in number per pot were removed. The system of replication consisted of duplicate pots of each treatment. That is to say, measurements of disease were made on a basis of 24 plants.

The plants were allowed to develop for 15 days from the time of seeding, and, throughout this interval, the pots were weighed daily and, when necessary, more water was added to maintain the water content of the soil at 40% saturation. An effort was made to keep glass-house temperature within a narrow range of variation. At the end of this interval, the plants were in the three-leaf stage and, in the controls, there was a profuse root development. The entire plants were removed together with the core of soil from which they were separated by gentle washing in a lead of water over a sieve. Each plant was then examined in a water-bath for evidence of disease. In estimating the amount of disease caused by the different isolates, the disease rating method developed by Greaney (5) was adopted, with this difference that the infection classes comprised nine instead of six classes. This provided a better opportunity for weighting the size and position of the lesions.

EXPERIMENTAL RESULTS

In the first experiment, the soil was steam sterilized at 15 lb. pressure for four hours. Ten of the isolates earlier mentioned were tested. The range of temperature was from 16.2° to 24.2° C., with a mean of 19.2° C. Later this test was repeated, the temperature range being 15.5° to 23.3° C., the mean 18.8° C.

In the second experiment, a comparison was made of the pathogenic behaviour of nine of the isolates in unsterilized soil and in unsterilized soil with the addition of 1% grass-meal. The temperature during the experiment ranged from 17.5° to 25.3° C., the mean being 19.9° C. The results of this experiment are included in Table III.

TABLE III

THE EXTENT OF PARASITISM OF *R. Solani* ISOLATES FROM WHEAT IN DIFFERENT SOILS

(Data are means of two trials)

Isolate	Experiment 1		Experiment 2			
	Ratio of inoculum to soil					
	Sterilized soil		Unsterilized soil		Unsterilized soil + 1% grass-meal	
	1 : 6	1 : 3	1 : 6	1 : 3	1 : 6	1 : 3
<i>S.</i> 22	6.8	4.7	12.6	9.6	12.0	8.1
<i>M.</i> 2	7.8	3.4	8.1	7.4	5.8	4.4
<i>M.</i> 3	13.0	10.4	19.2	13.3	15.5	11.1
<i>M.</i> 4	14.7	11.5	17.0	11.1	14.9	14.0
<i>S.</i> 23	15.4	13.2	20.5	17.0	13.3	11.8
<i>S.</i> 21	9.2	6.8	—	—	—	—
<i>B.</i> 5-3	1.6	0.5	5.1	3.7	1.5	1.5
<i>B.</i> 140	11.1	7.8	16.3	9.4	12.5	8.8
<i>R.</i> 5	31.8	30.9	42.2	38.3	28.3	24.3
<i>R.</i> 6	29.6	26.2	43.7	35.5	32.2	31.2
Controls ¹	0.0	0.0	0.3	0.4	0.4	0.7

¹ Average disease rating for all the controls in each test.

The results of the tests with two different proportions of inoculum added to the soil (Table III) indicate that, with all isolates and in all soil treatments, the disease rating was higher in the soil to which inoculum was added at the rate of one part to six parts of soil than in the soil to which inoculum was added at the rate of one part to three parts of soil. In this, the results are in agreement with those of Sanford (16) who found that there was a decrease in the virulence of isolates of *R. Solani* from potato as the amount of inoculum added to the soil was increased over the proportion, 1 : 15. In so far as the influence of the substrates is concerned, it is evident that each isolate was more virulent in the unsterilized than in the sterilized soil. Sanford (16) obtained similar results with his isolates from potato. In the present investi-

gation, the addition of an organic supplement, grass-meal, reduced the amount of infection in the soil to which it was added, as compared to that in soil devoid of this supplement.

In the examination of the plants, however, it became evident that the disease ratings determined by the above mentioned method do not express the most striking development of the parasitism, namely, that there were two quite different symptomatic expressions of *R. Solani* infection. The isolates *R. 5* and *R. 6*, which had been isolated by the writer from wheat roots in England, constituted a distinct group from the other isolates under study, which were of Canadian origin. Isolates *R. 5* and *R. 6* caused a form of root injury ranging in severity from a slight root tip necrosis, through degrees of brown lesioning of the intermediate portions of the root system, to an extremely severe expression of disease in the form of almost complete destruction of the entire primary and secondary roots. Fig. 3 depicts this condition in infected barley plants taken from a field in Norfolk, England, and illustrates exactly the condition produced by the isolates *R. 5* and *R. 6* on wheat plants.

The characteristics of this type of injury include a killing of the root tips, with a stimulation in the formation of laterals, which in turn are attacked. This type of injury seems identical with the *R. Solani* root infection described by Samuel and Garrett (12) on cereals, under South Australian conditions. The injury markedly affected the vegetative growth of seedling plants, for they were spindly and stunted, with a tendency to leaf rolling.

On the other hand, the isolates of Canadian origin produced quite another type of injury. In this type, there was only a light brown lesion, varying in size from a speck to a length of over 1.5 cm., on the coleoptile above the kernel (Fig. 2), and hence is referred to as "stem girdling" injury. Furthermore, this type of injury did not appear to affect adversely the seedling development of the plants.

It was apparent from the evidence of histological examination of cotton-blue-lactophenol preparations of affected tissue that in the case of the root injury type of disease caused by *R. 5* and *R. 6*, the parasitic hyphae had ramified throughout the cortical and vascular host tissue causing complete disintegration. But with the stem girdling injury, the fungus hyphae appeared only to be aggregated in the epidermal tissue and an examination of macerations of the cortex from such affected stems did not indicate that the hyphae had penetrated that tissue (Fig. 4).

Typical plants demonstrating either root injury or stem girdling, were retained in inoculated soil and examined throughout later growth stages. Those plants, which in the seedling stage had been affected with coleoptile girdling lesions, were found after examination in the mature condition still to bear *R. Solani* infection in the form of a girdling lesion only on what remained of the coleoptile encasing the subcrown internode. The root systems of these plants were normal in comparison with control plants, and there was no evidence of root damage. Plants maturing from seedlings affected with the root injury form of disease were still characterized by the

seedling type of infection—a necrosis of tissue, amounting in extreme cases to complete stunting of the root system. In these cases there was, however, no sign of parasitic infection of the lower stem tissue.

In view of the fact that the English isolates *R. 5* and *R. 6* produced disease symptoms of wheat seedlings quite distinct from those resulting from the parasitic action of the Canadian isolates of *R. Solani*, it would seem that the use of one disease rating scale, covering two different forms of injury, is not warranted. In the third experiment, a separate disease rating scale was applied to the two forms of parasitism. The classes used in each are shown in Table IV.

TABLE IV

CLASSES, NUMERICAL RATINGS, AND FORMULA¹ FOR COMPUTING DISEASE RATING USED TO RECORD THE DEGREE OF INFECTION OF *Rhizoctonia Solani* ON WHEAT PLANTS

Class	Degree of infection on individual plants	Numerical rating
<i>Numerical ratings for plants affected with stem girdling lesions</i>		
1	No infection; roots and stems normal	0
2	One to several small coleoptile lesions (less than 0.5 cm.); no root injury	1
3	Single coleoptile lesion (0.5 to 1.0 cm.); no root injury	2
4	Single coleoptile lesion (1.0 cm.); no root infection	3
5	Single coleoptile lesion (1.5 cm.); no root infection	4
6	Coleoptile lesions greater than 1.5 cm.	5
<i>Numerical ratings for plants affected with root injury</i>		
1	No infection; roots and stems normal	0
2	Slight injury to root tips	1
3	General distribution of small brown disease lesions throughout the root system without any apparent reduction in root length	2
4	Destruction of roots from apical points, leaving short proliferating root stumps from 2.5 to 4.0 cm. in length	3
5	Roots severely reduced, leaving stumps less than 1.3 cm. long	4
6	Almost complete destruction of the roots	5

$$^1 \text{Disease rating} = \frac{\text{Sum of numerical ratings} \times 100}{\text{Number of plants examined} \times \text{maximum rating}}$$

The third pathogenicity experiment was arranged in the same manner as the two described earlier, but, in it, sterile and natural soil were used concurrently, not successively as in former tests. In this experiment, the organic supplement of 1% straw-meal was included instead of grass-meal. During

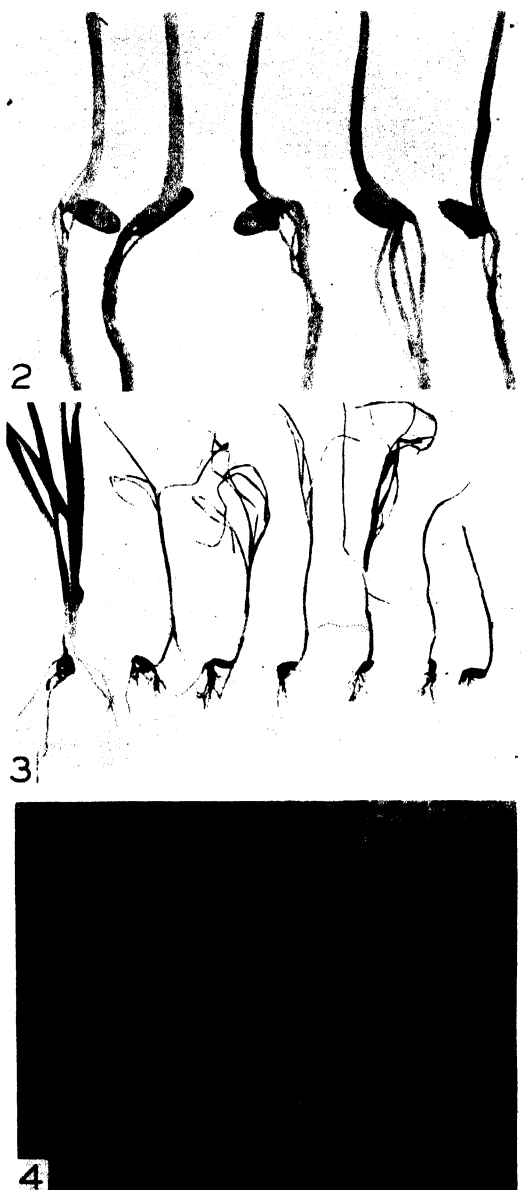


FIG. 2. Infection types of stem girdling injury caused by the *R. Solani* isolates of Canadian origin; numerical ratings (left to right): 0, 1, 2, 3, and 4.

FIG. 3. The type of root injury caused by two *R. Solani* isolates (*R. 5* and *R. 6*) of English origin; six infected barley plants, control plant on extreme left.

FIG. 4. Microphotograph showing the distribution of *R. Solani* hyphae in infected coleoptile tissue of wheat. $\times 400$.

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the experiment, the temperature ranged from 18.9° to 26.0° C., with a mean of 20.1° C. The results are shown in Table V, the disease ratings being derived from use of the scales given in Table IV.

TABLE V

THE EXTENT OF PARASITISM OF WHEAT ISOLATES OF *R. Solani* IN NATURAL AND STERILIZED SOIL AND IN NATURAL SOIL PLUS 1% OF STRAW-MEAL

Isolate	Ratio of inoculum to soil					
	Sterilized soil		Unsterilized soil		Unsterilized soil + 1% straw-meal	
	1 : 6	1 : 3	1 : 6	1 : 3	1 : 6	1 : 3
Disease rating for stem girdling injury						
<i>S.</i> 25	30	20	46	40	29	21
<i>M.</i> 4	36	30	45	35	32	27
<i>M.</i> 2	34	28	41	32	26	19
<i>S.</i> 22	35	29	46	40	23	20
<i>B.</i> 140	36	29	49	42	39	29
<i>S.</i> 21	40	31	53	47	30	22
<i>S.</i> 23	43	31	48	43	39	25
<i>M.</i> 3	40	36	43	37	32	28
Controls ¹	0.0	0.0	2.6	2.4	3.4	3.2
Disease rating for root injury						
<i>R.</i> 5	42	36	44	41	24	19
<i>R.</i> 6	35	31	48	44	21	16
Controls ¹	0.0	0.0	2.0	2.5	2.0	1.5

¹ Average disease rating for all the controls in each test.

The results of this experiment are in close agreement with those presented in Table III. For each isolate, the disease rating was higher in the soil to which inoculum was added at the rate of one part to six parts of soil than in the soil to which one part of inoculum was added to three parts of soil, regardless of the soil treatment. Similarly, there was, for each respective rate of inoculum, a higher disease rating in the unsterilized than in the sterilized soil. Furthermore, in comparison with that in unsterilized soil, the disease rating was lower in unsterilized soil to which an organic supplement, in this instance, straw-meal, was added.

Discussion

Throughout these experiments, the disease ratings for those isolates producing a stem girdling injury, indicate that differences exist in the ability of the individual isolates to act as parasites. It appears, for instance, that isolate *B.* 5-3 is only a weak parasite (Table III). On the basis of the nature of their parasitic action, all these isolates appear to be closely related, and may perhaps be regarded as belonging to one group. No differences could be detected in their microscopic or cultural characters.

Sharply distinct from the stem girdling injury is the severe root infection caused by the two English isolates *R. 5* and *R. 6*, both of which act identically. These two isolates obviously belong to a different group of *R. Solani*. When the symptomatic differences in the pathogenic action of the two groups were first revealed, it was felt that *R. 5* and *R. 6* might indeed not be *R. Solani* Kühn at all but rather an undescribed species of *Rhizoctonia*. This possibility received some support in that, regardless of the microscopic similarity of *R. 5* and *R. 6* to the Canadian isolates, macroscopically there were differences in the nature of the sclerotia. Instead of the massive dark brown sclerotia characteristic of the isolates causing stem girdling injury, the sclerotia of *R. 5* and *R. 6* were only pin-head in size and very light brown in colour. Nevertheless, in all cases where cultures of *R. 5* and *R. 6* were submitted to other authorities for examination and identification, the conclusion was that these isolates must be considered to belong to the "*R. Solani* group"¹. However, pending the discovery of the sporiferous stage of these two isolates, it is suggested that they be regarded as a variety of *R. Solani* Kühn.

Apparently under the conditions provided by natural soil, the inoculated organism derives some stimulus from a microbiological association factor which is lacking in the sterilized soil. It is possible that, as suggested by Sanford (16), *R. Solani* in the steamed soil is encouraged to develop a greater quantity of mycelium with a possible accumulation of staling products detrimental to parasitic action. Similarly, in the heavier but less pathogenically effective rate of inoculation (one part inoculum to three parts of soil), an increased density of mycelial growth may be associated with the lower incidence of disease. In his studies, Sanford (14, 15) has observed consistently that conditions encouraging very profuse growth of *R. Solani* usually result in a lower degree of parasitism and that his very profuse growing isolates are among the least pathogenic to the potato host. Further evidence that the extent of growth through the soil may not exhibit a positive correlation with the degree of parasitism, or, conversely, that the fastest growing isolates are not necessarily the most actively parasitic, is afforded by a comparison between the soil growth rate figures for isolates *R. 5* and *R. 6* (Table I), and the disease ratings just considered (Tables III and V). The isolates *R. 5* and *R. 6* are shown to have been the slowest growing isolates in the soil, but yet they were the most severe parasites.

Finally, in regard to the influence of the organic supplements, grass- or straw-meal at the rate of 1% (Tables III and V), it was found that each of these organic materials, with all isolates, caused a reduction in infection compared with that pertaining to soil not supplied with additional cellulosic organic matter. Earlier work in this investigation, an account of which will appear at an early date (2), has shown that these materials greatly inhibited the growth of *R. Solani* in soil. It would seem that those conditions associated

¹ The writer is indebted to the following workers who made a critical examination of cultures of *R. 5* and *R. 6*: G. R. Bisby, Imperial Mycological Institute, Kew; E. L. LeClerc, Louisiana Experimental Station; R. Weindling, South Carolina Experiment Station; N. M. Walker, Florida Experiment Station; J. E. Machacek, Dominion Laboratory of Plant Pathology, Winnipeg, Man.

with this growth inhibition of *R. Solani* in the presence of organic matter, including aspects of microbial antagonism and competition, nitrogen and phosphate deficiency, and carbon dioxide accumulation are also effective in reducing the parasitic vigour of this fungus in relation to wheat seedlings.

Acknowledgments

The writer wishes to record his deep appreciation of the stimulus and guidance of Mr. S. D. Garrett, Rothamsted Experimental Station, England. He wishes also to express his thanks to the Dominion Botanist for accommodation and facilities at the Dominion Laboratory of Plant Pathology, Winnipeg, Man., and also to various members of the Staff of that Laboratory for their interest and assistance in the work.

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ON THE FRUCTIFICATIONS AND NEW TAXONOMIC POSITION OF *DACTYLOTHECA PARALLELA* KIDSTON¹

BY NORMAN W. RADFORTH²

Abstract

Using the transfer and maceration methods, an analysis of the fructifications of *Dactylothea parallela* Kidston has been made to obtain a more adequate description of their structural features for the purpose of revising its systematic position.

The results of the analyses show that the fructifications, hitherto believed to be single sporangia inserted superficially on the lateral veinlets of the lower surface of the pinnules, are really stalked, three- to five-component synangia inserted in the marginal regions.

The spores extracted were generally smooth and thin-walled, measuring 19 μ in average diameter, and the number per synangium was calculated to be 10,000.

On the evidence produced, the plant cannot belong to the genus *Dactylothea*, and after a comparison with the forms that it most closely resembles it has been concluded that it should belong to the genus *Asterothea* which has been extended here to include the scolecopteroid types.

Its fructifications appear to be more marginal than those of all other *Asterothea* species, their orientation is comparable only with that in *A. hemitelioides*, and they resemble the scolecopteroid fructifications in that they are stalked.

It has accordingly been regarded as specifically distinct, and named *Asterothea parallela* n. comb.

Its affinities have been discussed, and the evidence seems to warrant its classification as a marattiaceous fern.

When the plant to be dealt with here was first described by Kidston (9), it was with some doubt that it was classified in the genus *Dactylothea*. However, at that time it was indicated that "the essential characters of the species seem to agree well with those of *Dactylothea*, in which genus, for the present at all events, it may find a convenient place". In view of the results of investigations made on the other two members of this genus (11, 12), it has become increasingly important that the taxonomic position of this, the last remaining member of the genus, should be more accurately determined. In order that this may be accomplished, however, a more adequate description of the fructifications of the plant is required, and it was primarily for this reason that the present investigations were made.

Material and Methods

The piece of the frond of *Dactylothea parallela* Kidston that was studied in this work was preserved in the form of a compression, and was the specimen on which Kidston based his description of the plant. It was discovered in the Kiltonge Coal of the Lanarkian Series, and with its counterpart is the only specimen of its kind so far to be found.

¹ Manuscript received October 27, 1941.

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The methods used in analysing the structural features of the plant were similar to those described elsewhere (11, p. 385; 12, p. 746). The general shape, size, and arrangement of the pinnules, and occasionally the position of the fructifications could be observed from the frond as it appeared on the surface of the rock. These features, however, and in addition the arrangement, shape, size, and attachment of the fructifications could best be observed from the compression when it had been transferred to a cellulose film. Finally, the actual structural details of the fructifications, and the nature of their contents were revealed in an examination of the oxidized fragments obtained from the transfers which had been macerated with Schultze's solution.

Observations

In a superficial examination of the frond as it appears on the rock surface, its general habit is seen to be similar to that of the other members of the *Dactylothea-Senftenbergia* group (Fig. 1), and since this has already been accurately described by Kidston (9, p. 398) further remarks in this connection are unnecessary here. The position of the fruiting bodies, which are arranged with their long axes parallel, one row of them along each side of the under-surface of each pinnule, is shown in Fig. 2. In this photograph, which represents part of an ultimate pinna, since most of the remains of the pinnule laminae are almost entirely absent, practically all that remains are the sporangia which have retained their normal relative positions. In an enlarged view from this transfer, the margin of the pinnule, though fragmentary, is present as shown in Fig. 3*m*.

From this photograph most of the surface detail of the fructifications can be made out. They are elliptic, generally more or less obtuse at both ends, and their surfaces are coriaceous in appearance. Kidston (9, p. 399) reported that there is no evidence of a region of specialized annulus cells on any of the fructifications, and the observations reported here confirm his in this connection.

As a result of the transfer technique, however, two very important features revealed in this work do not agree with Kidston's results. The first is that the fructifications are attached by a stalk to the marginal region of the pinnule (Fig. 3*p*), not inserted in a strictly superficial position on the lateral veinlets of the pinnules. The method of attachment is clear in only two of the fructifications in this photograph. One of these has been photographed at higher magnification (Fig. 4) and the stalk is indicated at *p*. The second feature disagreeing with Kidston's description is that in nearly every case the fructifications appear to be divided into two or three lobes or folds (Fig. 3, 4*l*), suggesting a compound rather than a simple structure.

An examination of the macerations made from the transfers revealed no evidence of an annulus in any fructification examined, regardless of the length of the period of oxidation to which the material has been subjected, which varied from one to six days. The average length of a fructification, which

was determined from measurements of several isolated examples occurring in the mounts prepared from the macerations, is 0.6 mm., and the width is 0.4 mm.

Fig. 5 is a photograph of a fructification that has been in the oxidizing fluid for a period of four days. In this, and in other oxidized specimens, the lobing effect is more clearly observed than it was in unoxidized specimens. The spore mass inside the remnants of the sterile tissue is divided at the apex into finger-like projections. In the specimen that has been illustrated, two of these are visible, and it seems reasonable to suppose that a third projection was present at *p*, where only sterile fragments now remain.

The spores are thin-walled, either round or oval in shape, and are smooth or only slightly wrinkled. Where the wrinkling is most pronounced the spore wall is usually in a collapsed condition, a feature that is illustrated in the photograph of a spore group (Fig. 6). The spores, whether they are derived from the same sporangium or not, may vary considerably in size. The average diameter from a series of normal spores is 19 μ , and the approximate number of spores from one complete fructification has been calculated to be 10,000*.

The evidence from these analyses points to a view that the fructifications of *D. parallela* are really synangia composed of from three to five sporangia which in this stage of their development are free at their apices, and joined at their bases to a common stalk. The divided appearance of the fructifications in Figs. 3 and 4, especially where the lobing *l* has been indicated, strengthens this view. The fructification at the lower right in Fig. 3 has

* The method employed in estimating spore numbers was the same as that used in previous investigations (12, p. 746).

EXPLANATION OF FIGURES

With the exception of Fig. 3a, all figures are from untouched photographs.

FIG. 1. Part of the compression showing the general habit of the frond and the arrangement of the pinnales. Kidston Collection Specimen No. 2673. $\times 1$.

FIG. 2. Cellulose transfer of part of a pinna showing the position of the fructifications. Most of the carbonaceous material of the pinna limb is missing, but the outline of the margin is seen at *m*. $\times 5.5$.

FIG. 3. Two rows of fructifications from part of the lower surface of a pinna, photographed from the transfer (Fig. 2) with the aid of an ultrapack; *p* = stalk; *m* = pinna margin; *l* = lobing of the fructifications; *x* = fructification compressed in the upright condition. $\times 30$.

FIG. 3a. Outline drawing of the fructification seen at *x*, Fig. 3, clarifying the lobed condition which suggests a five-component synangium. $\times 30$.

FIG. 4. An enlarged view of a single fructification as it appeared on the transfer, photographed with the aid of the ultrapack, showing lobing, *l*, and the stalk, *p*. $\times 60$.

FIG. 5. A fructification that had been oxidized in Schultze's fluid for four days, showing the lobing effect and the mass of spores contained within the sterile remnants; *p* = projection of a sterile portion of a lobe of the fructification. $\times 125$.

FIG. 6. Group of spores extracted from a mass similar to that in Fig. 5, showing the character of their walls and their variability in size; *t* = triradial ridge. $\times 535$.





been compressed by a force acting in a direction parallel to its long axis, and appears in the form of a closed rosette. Here, with a little care, five lobes can be discerned; the apices of these come together at a point near the centre of the rosette. In order to clarify this condition an outline drawing of the sporangium is provided adjacent to the photograph, with the lobes or single sporangia numbered around the edge (Fig. 3a).

If the fructification of this plant were to be regarded as a single sporangium, then the calculated spore output of 10,000 would be very high. If, on the other hand, it is regarded as a five-component synangium where each constituent sporangium is equal in capacity to that of its neighbour, the spore count, when computed on the basis of the number of spores per sporangium, falls between 1500 and 2000. With the single exception of *Ophioglossum pendulum* (2, p. 262), it is within these limits that the spore count of the living syngiate ferns characteristically occurs. The structure and variability in size of the spores of the same sporangium suggest that they were in an immature stage of development at the time of fossilization. If this were true, the spore walls would probably have been relatively more glutinous than they would if the spores had been older. Accordingly, they would have tended to adhere to each other and to the walls of the sporangium. This condition was frequently found to occur, and is illustrated in a typical example (Fig. 5) where the spore mass still adheres to tapetum-like remnants of the sterile parts of the sporangium.

Discussion and Conclusions

In the remarks given by Kidston (9, p. 398) justifying the placing of this plant in the genus *Dactylothea*, it was indicated that the description of the species agreed with that of the genus "in the position of the sporangia on the veins, in their general form and structure (though their arrangement on the pinnules is more regular in forming a single row on each side of the midrib), and in their occupying the whole of the limb between the median vein and the margin". From the observations reported here, this statement can no longer be supported. Hitherto, the point of insertion of the sporangia in *Dactylothea* has been regarded as being superficial in position; in *D. parallela* the writer's observations show that although the fructifications are attached on the abaxial side of the pinnule, the point of insertion is distinctly in the marginal region of the limb. Also, in view of the evidence given here, the fructification, which Kidston regarded as being a single sporangium, is really a sorus of sporangia. These newly discovered features are not only contrary to those included in the description of the genus *Dactylothea* Zeiller (16), but provide, in addition, evidence that precludes any justification for believing that there is any immediate relationship between this species and the genus *Dactylothea*.

For the same reasons, and also because the sporangia are exannulate, it is obvious that this plant is not to be classed within the genus *Senftenbergia* to

which two other forms, *Dactylothea plumosa* and *D. Sturi*, have recently been transferred (12).

There are, however, three other genera that have certain diagnostic features in common with those found in this species. These are *Asterotheca* Presl, *Acitheca* Schimper, and *Scolecopteris* Zenker. All of these, as well as the species being considered here, resemble each other in the essentials of synangial structure. In each case the synangia are arranged in a row down each side of the pinnule limb, and are each composed of at least three, and as many as five, sporangia that are free at their apices and joined at, or near, their bases. None have annulate sporangia. Despite these common likenesses a few differences do exist by which the fructifications may be distinguished, and before the correct systematic position of *D. parallela* can be fixed it will be necessary to compare its new diagnostic features with those of the other three similar types.

First, the position of the synangia on the pinnule limb is a feature worthy of some consideration. Out of 10 species of *Asterotheca* that Kidston reviewed (9), only one, *A. crenulata*, has marginal sporangia; the other nine have sporangia that are usually inserted about midway between the margin and the midvein of the pinnule. In *Acitheca* and *Scolecopteris* the synangia are unquestionably superficial in position, since a portion of the limb that separates them from the pinnule margin curves back and partially covers the synangia, serving as the protective flap (9, pp. 536, 537; Text-figs. 63,65). Thus, *Asterotheca crenulata* and *Dactylothea parallela* are the only two plants with marginal synangia and, in a comparison of these two, the synangia of the latter are closer to the margin than those of the former (cf. Fig. 2, of this work, and 9, p. 519, Text-fig. 59).

Another feature distinguishing *D. parallela* from these other types is the orientation of the synangia with respect to the pinnule surface. The synangia in *Acitheca* and *Scolecopteris* are situated on the pinnules in such a way that the long axes of the sporangia are perpendicular to the surface of the limb. In all but one species of *Asterotheca* the sporangia also stand upright. The species in which this condition does not occur regularly is *Asterotheca hemitelioides*, and according to Kidston (9, p. 522) synangia of this plant are sometimes found lying parallel to the surface of the pinnule as in *D. parallela*. In Kidston's opinion the horizontal position is considered as being "probably the result of accident", but in *D. parallela*, where the synangia almost invariably lie parallel to the pinnule surface, this condition seems to be the normal one, and the upright condition to be accidental since it occurs so infrequently. Admittedly, it seems highly probable that the synangia of *D. parallela* were pendant in their living condition, like those of *A. hemitelioides*, but their structural relationship with the pinnule lamina must have been different from that in *A. hemitelioides* in that the horizontal orientation has been imposed in fossilization to such a marked degree.

A third feature that offers a means of comparison is the method by which the synangia are attached to the pinnule limb. In the types under discussion

attachment may be according to one of three ways: the synangia may be inserted directly on the pinnule surface, or they may be attached by means of a receptacle, or by a short stalk. The synangia of *Acitheca* are attached as in the first of these three ways (9, p. 536), those of *Asterotheca* as in the second (9, p. 538, Text-fig. 66), and those of *Scolecopteris* (9, p. 535) and *D. parallela* (ante, p. 187) as in the third method. In this respect, then, *D. parallela* resembles *Scolecopteris*.

Finally there remains a consideration of the size and shape of the fructifications. For convenience in comparison, the synangial dimensions for appropriate representative species are recorded below.

Species	Length of synangium, (mm.)	Width of synangium, (mm.)
<i>Asterotheca crenulata</i>	0.75	0.40
<i>Asterotheca hemitelioides</i>	0.75 to 1.0	0.30 to 0.40
<i>Scolecopteris oliveri</i> *	1.60	0.44
<i>Acitheca polymorpha</i> **	2.50 to 4.0	0.50 to 0.75
<i>Dactylothea parallela</i>	0.60	0.40

* Reference 15, p. 8.

** Reference 13, p. 157.

It may be seen that the synangium of *D. parallela* is smaller than those of the other species. The synangium of *Scolecopteris oliveri* is considerably larger than that of *D. parallela* with which it compares favourably as to mode of attachment, and that of *Acitheca polymorpha* is larger still. The synangia of *D. parallela* most closely approach in size those of *Asterotheca crenulata* and *A. hemitelioides*.

An adequate comparison of spore size for these plants cannot be constructed because the spore dimensions for the two species of *Asterotheca* have not been determined. Moreover, the accuracy of the comparison is dependent upon the condition that the spores to be compared are of corresponding stages in development, which does not apply in this case. It is of importance to note, however, that the spores of *Scolecopteris oliveri*, which are ornamented and therefore presumably advanced, are 18 μ in diameter, where those of a *D. parallela*, which are presumably young, are 19 μ in diameter. If the spores of these plants were in corresponding stages of development there would probably be a considerable difference in their sizes, those of *D. parallela* exceeding those of *Scolecopteris oliveri*.

From these comparisons it is evident that the fructifications of *D. parallela* least resemble those of *Acitheca*, chiefly owing to their marginal position and horizontal orientation on the pinnule limb, their stalked condition, and their small size. In that they are stalked there is a resemblance to the type of synangium belonging to *Scolecopteris*, but here again there is disagreement as to position, orientation, and size. The condition as to position and structure

of the synangia in *D. parallela* is most closely approached in the genus *Asterotheca*, but only in the two species *A. crenulata*, where the synangia are almost as close to the margin of the pinnule as they are in *D. parallela*, and *A. hemitelioides* where they often lie parallel to the pinnule limb. Such likenesses, however, are confined to these two species of *Asterotheca* and, like all other plants of this genus, they have, by definition, sessile synangia.

Before deciding the new systematic position of *D. parallela*, with the help of these results, there is another factor that has an important bearing on the decision. Scott (15, p. 9) has already pointed out that it is difficult to identify forms preserved as petrifications with those preserved as compressions, and this is especially true in the comparisons that have just been made. Although a comparison of the petrified synangium of *Scolecopteris* with the carbonaceous synangium of *Asterotheca* indicates that these types have many features in common, the resemblance might have been even more obvious had both types been preserved in the same way. If the synangium of *Scolecopteris* had been fossilized as a carbonaceous compression, the pedicellate condition might not have been so evident since the stalks in all likelihood would be telescoped, and would have an appearance not unlike the small papillae on the pinnule limb in *Asterotheca* where the synangia are inserted. Whether or not the converse of this would hold is more difficult to decide. It seems reasonable to point out here that although the synangia in some, and perhaps in most of the species of *Asterotheca* might prove to be sessile if they were found uncompressed in the petrified condition, the possibility that some of them might be pedicellate still remains. Hitherto, the presence of the pedicel in the synangium of *Scolecopteris* has been made the main feature distinguishing the genus from *Asterotheca*, but in view of the theoretical conclusions reached above, the writer feels that there is no real necessity for placing both these types in separate genera and is inclined, with Hirmer (6, p. 576), to include them both in the genus *Asterotheca* Presl.

From the generic standpoint, there is now no doubt as to the new systematic position of *D. parallela*. Since its most important diagnostic features conform to the emended definition of *Asterotheca* Presl, it must be included within this genus. However, in the comparison of this plant with the others of the genus *Asterotheca*, there are still differences in degree in synangial structure, if not in type. Although, as it has already been pointed out, *Asterotheca crenulata* and *A. hemitelioides* bear some resemblance to *D. parallela*, the distinct marginal position of the synangia of the latter and their marked tendency to lie in a horizontal position on the pinnule limb, together with the presence of the stalk, are features that are sufficiently important to justify its position in a separate species.

The writer proposes that the plant now be designated as *Asterotheca parallela* (Kidston), and for convenience of identification or comparison with other types the following diagnosis is given:

Asterotheca parallela* (Kidston) n. comb.Dactylotheca parallela* Kidston

Fructifications in the form of elliptical synangia lying horizontally at either side of the midvein on the under surfaces of the pinnules, with their long axes parallel and pointing in a direction at right angles to the pinnule margin; attached at their bases by a short stalk to the pinnule limb in a marginal position. Sporangia in each synangium appearing to vary from three to five in number, united at their bases, and free at their apices. Average length of a synangium, 0.6 mm.; average width, 0.4 mm. Calculated number of spores per synangium, 10,000 (approx.). Average spore diameter, 19 μ .

In formulating this diagnosis there has been no mention of the few exceptional cases where synangia have been compressed in the direction parallel to their long axes. The omission was made because it was felt that this condition is not a typical one. Nevertheless, the condition is important since it points to the close relationship that this plant bears to the other species of *Asterotheca* where almost invariably the synangia have been compressed in the manner indicated. The other condition, the most typical one, where the synangia lie horizontal to the pinnule limb, is of equal importance because, owing to this orientation, the synangial stalk has been exposed and this, on the other hand, indicates a close relationship with the *scolecpteroid* forms. Thus, the description of this specimen of *Asterotheca parallela* since it includes both these conditions, serves to link that group of species conforming only to the old definition of *Asterotheca* with the group of species that have hitherto been confined to the separate genus *Scolecpteris*.

Affinities of Asterotheca parallela

Kidston (9, p. 547) pointed out that if the central column which unites the bases of the sporangia in *Acitheca* were extended for the full length of the sporangia, the result would be the formation of a solid synangium comparable with that of *Ptychocarpus*. It is interesting to note that a condition somewhat similar to this would be obtained if the components of the synangia of *Asterotheca parallela* were united along their full length instead of just in the basal region. Both Bower (1, p. 521) and Scott (14, p. 253 to 254) lean strongly to the view that by reason of its synangial structure *Ptychocarpus* shows strong marattiaceous affinities, and they indicate that among the genera of this family *Kaulfussia* has the synangia with which those of *Ptychocarpus* compare most favourably. Since the fundamental differences between the *Asterotheca parallela* type of synangium and the *Ptychocarpus* type are so few, it seems reasonable to suggest that the former of these should now also be considered when discussing the possible origin of the *Kaulfussia* type of synangium.

The marattiaceous type of synangium has also been referred to that of *Anachoropteris* for comparison (3, p. 39) and in the particular case of *Anachoropteris pulchra*, the structure of which has been worked out in detail by Kubart (10), the synangium shows strong evidence of marattiaceous affinities. This fructification is also fundamentally similar to the fructification of *Asterotheca parallela*, more especially since it is marginal in its position on the pinnule. The pinnule, however, is not of the pectopteroid type, and it would seem that

any resemblances in the structure and position of these synangia have been brought about as a result of parallel evolution, and are not marks of close relationship.

Before discussing the affinities of *A. parallela* at further length, two conditions that have an important bearing on this question should be considered. The first of these has to do with the stage in development of the synangium at the time of fossilization. It has been suggested (*ante*, p. 189) that from an examination of the spores the sporangia are in an early developmental stage. If such is the case there is the possibility that had they matured they would have separated and become almost totally free, which is the condition in some of the other species of *Asterotheca* (6, p. 579). While synangia of this type still resemble those of *Kaulfussia* as to their rounded shape, the relationship of the sporangia to each other is not unlike the corresponding condition in *Angiopteris*.

The second point that has a bearing on the question of the affinities of *Asterotheca parallela* is one commonly considered wherever fossil marattiaceous-like fructifications have been discussed. It concerns the possibility that these fructifications might be the microsporangia of Pteridosperms. Such a view receives support in this case for three reasons. One is that in general the types of synangia under discussion here seem to differ only in detail of structure from the microsporangia of *Telangium* (9, p. 538). Another is that Halle (5, p. 5) has described a pectopteroid frond, *Pecopteris Wongii*, which has a seed attached to its main rachis, and which is barely distinguishable from a frond of *Pecopteris* (*Asterotheca*) *Milioni*, a plant bearing the usual *Asterotheca* type of synangium, and this leads to an assumption that the *Asterothecas* may have been Pteridosperms. Before Halle's work was published, Hoskins (8) referred to this discovery, and in his account of the fructification of *Scolecopteris minor* expressed the possibility that this too may have been the male fructification of a Pteridosperm. Finally, Hirmer (7) in a detailed comparison of *Crossotheca pinnatifida* and *Asterotheca truncata**, showed on good grounds from the structure, position, and relationships to the pinnules of the fructifications of the latter species, that it appears to represent an intermediate type between the other species of *Asterotheca* and the species of *Crossotheca* which are undoubtedly Pteridosperms.

From these arguments there are grounds for supposing that *Asterotheca parallela* might also be a Pteridosperm, but, on the other hand, it has been shown that there is good evidence supporting the view that it is a fossil marattiaceous fern. Certainly, no seeds have been discovered in association with it, but this is the only specimen that has been found, and the possibility of discovering fronds bearing seeds cannot be excluded. The evidence

* On the elongated form of pinnule in *Asterotheca truncata* the synangia are situated very close to the midrib, but wherever the pinnule is fertile the lamina seems to be vestigial (4, Heft 4, Taf. XVII, Figs. 2, 5) and it does not extend over the synangia. Thus, the synangia appear to be marginal but, had the pinnule limb been extended laterally to its normal limit, the synangia would be distinctly superficial as they are on the shorter, more distal pinnules (*loc. cit.*, Fig. 3). Their apparent marginal condition, then, is not comparable to that in *A. parallela* where the synangia are not only normal but also situated on normally shaped pinnules.

produced in this work cannot serve to settle this dilemma, and any conclusions as to the ultimate relationships of the plant must be almost purely a matter of opinion. The writer can see no clear basis for the view that the species of *Asterotheca* in general should be regarded as Pteridosperms, as Kidston seemed to do. In the particular case of *Asterotheca parallela*, it cannot be denied that the fructifications are definitely marattiaceous-like, and since there is no direct evidence for supposing the plant to be a Pteridosperm it would seem logical to regard it, at least tentatively, as a marattiaceous fern.

Acknowledgments

The author wishes to thank Professor J. Walton of Glasgow University for the helpful advice he gave while these investigations were being made, and Dr. R. Crookall of the British Geological Survey for obtaining permission to investigate the fossil, which is specimen No. 2673 of the Kidston Collection. He is also grateful for the help of Dr. W. A. Bell, Canadian Geological Survey for his co-operation in reading the manuscript.

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THE RESPIRATORY AND RIPENING BEHAVIOUR OF THE TOMATO FRUIT ON THE PLANT¹

BY K. A. CLENDENNING²

Abstract

The respiratory behaviour of tomato fruits during their growth and ripening on the plant has been studied in relation to the season and light intensity during growth. Growth was found to be associated with an absolute increase in respiration rate. Fruits of all populations showed a marked rise and fall in respiration rate during ripening. This senescent drift was confirmed in detail by following the respiration of individual fruits ripening while still attached to the plant. Although exhibiting the usual senescent drift as they changed colour, fruits grown in summer cloth house plots showed a consistently lower rate of respiration than that observed in all other populations. It also has been shown that the chief path of gaseous exchange of the mature attached fruit is localized at the stem end.

The present investigation was undertaken as one aspect of a study of the metabolism of the tomato fruit in development and storage. Information has been published recently (3) on the respiratory and ripening behaviour of the stored fruit in relation to the composition of its internal atmosphere. The present paper is concerned with the respiratory behaviour of the attached fruit in relation to the season and light intensity during growth. The area of free gaseous exchange also has been demonstrated to be at the stem end as in the stored fruit. Several biochemical aspects of the physiology of the tomato fruit in development and storage will be dealt with in subsequent reports.

Materials and Methods

The experimental material consisted of fruits of the Grand Rapids variety grown during 1938 at the Department of Botany, University of Toronto, and during 1938-1940 at the Department of Horticulture, Ontario Agricultural College. A comparison of populations grown at different seasons of the year necessarily involved a study of a series of greenhouse populations. The temperature was not allowed to drop below 60° F. but the atmospheric factors of the houses were not otherwise controlled.

¹ Manuscript received November 18, 1941.

Contribution from the Department of Horticulture, Ontario Agricultural College, Guelph, Ont., with the co-operation of the Department of Botany, University of Toronto, Toronto, Ont. This paper is based on part of a thesis submitted to the Graduate School of the University of Toronto in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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During the summer of 1938, an ordinary field plot also was grown alongside a field cloth house plot in which the light intensity was reduced by 50% as measured by a Weston phototronic foot-candle meter. In the following summer, populations were grown simultaneously in a greenhouse bed, in an ordinary unshaded outdoor bed, and in a cloth house bed shaded to 50% light intensity. To simulate the soil conditions of the greenhouse population, the shaded and unshaded outdoor populations were grown in the same depth of standard soil as was used in the greenhouse; also, the outdoor beds were isolated from adjacent soil by a 12 in. layer of washed cinders.

Measurements of respiration rate were made by the Pettenkofer tube method, employing a continuous air flow of approximately 1500 cc. per hour. As a standard temperature, 12.5° C. was adopted for initial respiration rate determinations while 18.3° C. was used in studies of the carbon dioxide emission of attached fruits. The air stream was drawn through soda lime towers to remove atmospheric carbon dioxide. It then was bubbled through 18% potassium hydroxide to adjust its relative humidity to 85% before entering the individual respiration chambers.

It has been shown that the tomato fruit passes through a growth cycle consisting of a phase of cell division followed by a phase of cell enlargement. The former is of brief duration, persisting only for about one week from setting and apparently it results in the production of a cell population that is roughly uniform in number from fruit to fruit (4). The latter phase, a matter entirely of the enlargement of existing cells, persists until the mature green stage is reached, after which the fruit undergoes little change in volume or weight. It is therefore clear that the calculation of respiration rates on a fresh weight basis from the mature green stage onward through the ripening phase yields data that not only describe the changes in rate per unit of gross mass of living system but also provide a useful index to the changing activity of the individual fruit and even of the average cell. The same obviously cannot be said of the stages antecedent to maturity. At successive stages of the enlargement phase, unit fresh weight (e.g., 10 kg.) represents not only decreasing numbers of fruits but also proportionately decreasing numbers of cells. Hence respiration per unit fresh weight during the growth cycle reveals nothing of the changes in progress per fruit or per cell. Indeed, since these changes are sometimes in the opposite sense to the changes per unit fresh weight, the observer may be misled in matters of physiological interest by reasoning exclusively from data that involve the one method of expression alone. The data for the growth phases are accordingly presented here both on a unit fresh weight and on a unit fruit basis.

Experimental Results

Changes in Respiration Rate Associated with Growth

Earlier authors have agreed that at successive stages of growth, increasing size of the fruit is associated with a diminishing rate of respiration until a minimal rate is attained just prior to the onset of ripening (1, 5, 8). These

conclusions, however, were based on respiratory data calculated on a unit fresh weight basis.

To demonstrate the drift in respiration associated with the last four weeks of growth, the initial rate of respiration of fruits picked when "early growing green" (8), and "mature green", was determined individually on 12 fruits of each class. The average fresh weight of the early growing green class, picked at an age of three weeks, was 22.3 gm. while that of the mature green fruits, seven weeks old, was 83.4 gm. All these fruits were from a 1940 early summer greenhouse population. Immediately after detachment and cooling to the storage temperature, the early growing green fruits showed an average rate of respiration of 4.7 cc. carbon dioxide per fruit per day at 12.5° C. The mature green fruits on the other hand respired at an average rate of 9.6 cc. carbon dioxide per fruit per day under the same experimental conditions. Thus during the final four weeks of growth, there was approximately a 100% increase in rate of carbon dioxide emission per fruit. When calculated on a fresh weight basis, the same fruits showed the downward drift recorded by earlier authors; the early growing green fruits had an initial rate of 87.3 cc. carbon dioxide per 10 kg. fresh weight per hour while the mature green fruits had a rate of only 48.0 cc. per hour on the same fresh weight basis.

Changes in Respiration Rate Associated with Ripening

The respiratory behaviour of the tomato fruit ripening in storage is unique in that the expected senescent rise and fall in carbon dioxide output may be abolished completely by appropriate restriction of gaseous interchange at the stem scar area (3). Several authors have claimed that the tomato fruit always shows a marked rise and fall in respiration rate as it ripens on the plant, but for the most part their observations were limited to populations grown at one season of the year (1, 5, 7, 8).

The initial respiration rate of fruits picked when mature green, yellow orange, and full red has been determined in the course of the present work for populations grown at different seasons of the year and under different light conditions during the summer. In each population, it was evident that passage through the ripening colour change on the vine involved at first a rise in respiration to the yellow orange stage, followed by a decline as the fruits ripened to full red (Table I). The season in which the fruits were grown showed no clear effect on the extent of these respiratory changes. With the exception of the cloth house or midsummer 50% light plots, the data in fact conformed closely with those published by Walford (8) for respiratory changes during ripening in situ. The cloth house fruits showed a parallel rise and fall in respiration during ripening but their level of respiration was consistently lower.

In common with all previous studies of this kind on the tomato fruit, the above work was limited to determinations of the initial rate of respiration at successive stages of ripening. To obtain a more detailed picture of the general respiration sequence established by initial rate determinations, con-

TABLE I

THE MEAN INITIAL RESPIRATION RATE OF FRUITS OF SEVERAL POPULATIONS PICKED BEFORE, IN THE MIDST OF, AND AFTER THE RIPENING COLOUR CHANGE. RESPIRATION EXPRESSED AS CC. CARBON DIOXIDE PER 10 KG. PER HOUR

Populations	Mature green	Yellow orange	Full red
1939, spring greenhouse	47	114	71
1939, late summer greenhouse	49	76	48
1939, cloth house	40	54	29
1940, early summer greenhouse	49	97	83
1940, midwinter greenhouse	52	97	57

tinuous measurements of respiration were made on fruits ripening while actually attached to the plant (Fig. 1A, B, C).

It should be noted that the only study of this kind that had been attempted previously on fruits was that of the apple reported by Kidd and West (6). In that study, the respiration chamber was opaque so that the colour changes could not be observed and the fruit became detached at some unknown time in the course of their experiment.

In the present study, a tomato plant that had set six trusses of fruits was transferred to a storage room maintained at a temperature of 18.3° C. Large glass jars fitted with three hole rubber stoppers were used as individual respiration chambers for the attached fruits. The stoppers were pared to a depth of one-half inch. The central hole in each stopper was of such a size that it would fit snugly around the pedicel without damaging its tissues. To insert the pedicel, the stopper was slit from its outer edge to the central hole. By holding this incision open it was possible to place the pedicel in the hole and on closing the incision a fair seal was obtained by the pressure on the cut rubber faces. The other two holes of the stopper took the inlet and outlet tubes for the air stream. The stopper then was fitted snugly into a glass jar and was smeared carefully with vaseline until tests showed the respiration

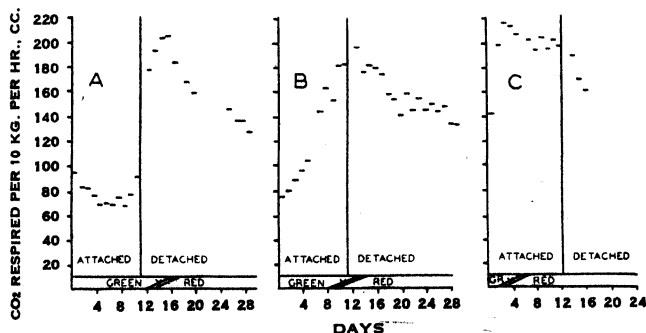


FIG. 1. The respiration at 18.3° C. of the tomato fruit on the plant, with detachment at the onset (A), at the peak (B), and on the declining arm (C), of the respiratory climacteric.

chamber to be airtight. Measurements of carbon dioxide output were taken by the usual continuous stream Pettenkofer method. Observations of ripening coloration were recorded daily. Finally the fruits were removed and weighed so as to allow calculation of the respiration data on a final fresh weight basis.

During the course of this experiment, the leaves of the plant turned yellow and after 12 days many of the leaves were brown. Because of this unhealthy condition of the leaves, the fruits in the respiration chambers were detached from the plant by severance of the pedicel (Fig. 1A, B, C), the respiration air stream being stopped only temporarily.

Fig. 1A, B, and C remove all doubt as to the reality of the climacteric rise in situ for the tomato fruit. The exact time of detachment in the present experiment is indicated and it is quite apparent that the act of isolation induces no significant change in the respiratory drift.

The respiration rate at 18.3° C. before the onset of the senescent rise approximated 70 cc. of carbon dioxide per 10 kg. per hour. The carbon dioxide output rose considerably before ripening coloration became evident externally. The appearance of orange colour in the outer tissues under these experimental conditions marked the attainment of highest respiratory activity. Subsequently the rate drifted downward until the fruits were dead ripe.

In agreement with Walford (8) and Kidd and West (6), it is clear from Fig. 1A, B, and C that the act of detachment does not interfere with the respiratory sequence which would have been exhibited by the fruit, had it remained on the plant. From this conclusion it should follow that the gaseous exchange at the stem end of the fruit which has been shown to be of such importance in the detached fruit cannot be influenced greatly by any partial plugging effected by the pedicel of the attached fruit. There is evidence that leaving a short piece of stem on the stored fruit results in a slight restriction of its gaseous exchange (2, 9) but the order of this restriction is by no means comparable to that observed when the stem scar area is waxed (2, 3). The drift in respiration rate of fruits in storage is essentially the same for those in which stems are removed as for those in which stems are attached (3). If the presence of the stem does not interfere greatly with gaseous exchange at the stem end of fruits either on the plant or in storage, the question arises as to the area through which the unobstructed diffusion of carbon dioxide and oxygen occurs. Do the gases from the fruit move through the pedicel or through other areas of the stem end? Or is the attached fruit characterized by a preponderance of cuticular diffusion in contrast with the detached fruit in storage?

By blocking diffusion at the stem scar area with wax, it has been shown elsewhere (2, 3), that the ripening of isolated mature green tomato fruits is seriously affected, the colour change always proceeding very slowly and unevenly. This alteration in ripening behaviour has also been shown to be a result of the changes induced in the fruit's internal atmosphere and is of

sufficient reliability to be used as an indication of the effectiveness of a particular waxing treatment in blocking gaseous exchange.

Copious application of vaseline or melted paraffin wax (175° F.) has been used previously with equal success in demonstrating that the gaseous exchange of the isolated fruit is localized principally at the stem scar area. In a study designed to indicate the path of gaseous exchange of the attached fruit these two substances have been used to block diffusion at different areas.

Five lots of attached mature green fruits were prepared in the following manner for observations of ripening behaviour:

- Lot 1. Calyx left attached and no other treatment given.
- Lot 2. Calyx carefully removed, otherwise untreated.
- Lot 3. Calyx removed and melted paraffin wax (175° F.) applied to the area of the fruit ordinarily covered by the calyx, to the parts of the pedicel immediately adjacent to the receptacle, and to the region of the receptacle itself in the angle between pedicel and fruit.
- Lot 4. Calyx removed and the pedicels heavily coated with vaseline from a few millimetres above the receptacle to the point at which the pedicel branches from the truss.
- Lot 5. Calyx removed and the skin of the fruit heavily coated with vaseline, leaving the pedicel and calyx area untreated.

The ripening behaviour of Lots 1, 2, and 4 was normal, all fruits attaining a full red colour within seven days from the time ripening commenced. The ripening of Lot 5 was also not inhibited but its treatment resulted in the appearance of brown blotches on the skin. The fruits that had been waxed at the stem end (Lot 3) on the other hand showed a marked inhibition of ripening rate, never attaining more than a pale orange colour. Twenty-one days after the first appearance of yellowing, all fruits of Lot 3 were not more than pale orange colour, although quite as soft as overripe fruit. With but one exception, the fruits of this lot dropped to the ground by natural abscission. The final appearance of the one fruit of this lot remaining in a state of attachment for 60 days was of particular interest. When picked after remaining on the plant for 60 days after copious waxing at the stem end, this fruit was mostly pale orange in colour but showed typical symptoms of blotchy ripening over the locules. Green coloration still was evident on the shoulder. There also was evidence of surface pitting (3) over one locule. Like the other fruits of this lot, softening progressed to a marked extent despite the inhibited ripening colour changes. It should be recalled that this physiological anomaly for which the term "premature softening" has been proposed has been observed frequently in fruits stored with stem scars waxed (3).

Thus the only treatment of attached fruits that caused an inhibition of ripening coloration was the application of wax to the stem end or calyx area. The area of free gaseous diffusion in the attached tomato fruit therefore must be localized at the stem end just as it is in the unwaxed fruit in storage. It further appears that in the attached fruit, the unobstructed path of gaseous

exchange is through that part of the fruit ordinarily covered by the calyx, or through the immediately adjacent parts of the receptacle and pedicel.

The restriction of gaseous exchange at the stem end of the fruit and the resulting alteration of the fruit's internal atmosphere inevitably leads to a marked inhibition of the ripening rate and usually to an unevenness in the colour attained either on the vine or in storage. These characteristic accompaniments of obstructed gaseous exchange are also symptoms of the common physiological disorders "blotchy ripening" and "leather end". The possibility of unusual local tensions of oxygen and carbon dioxide in the fruit flesh contributing to the appearance of these developmental disorders appears sufficiently feasible to warrant experimental investigation.

Acknowledgments

In conclusion, the author wishes to express his appreciation to Professor G. H. Duff and to Dr. J. H. L. Truscott for their constructive criticisms of the experimentation and manuscript. The author is indebted to E. Roy Waygood who conducted the respiration studies on attached fruits.

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VEGETATIVE PROPAGATION OF CONIFERS

XII. EFFECTS OF MEDIA, TIME OF COLLECTION, AND INDOLYL-ACETIC ACID TREATMENT ON THE ROOTING OF WHITE PINE AND WHITE SPRUCE CUTTINGS¹

By J. L. FARRAR² AND N. H. GRACE³

Abstract

Semimonthly collections of white pine and white spruce cuttings were taken from July to October, 1939, and propagated in several media. Collections of both species were taken in late October to examine the effect of type of cutting and of planting in media involving different proportions of two sands and two different peats. Cuttings were dusted with a series of concentrations of indolyl-acetic acid in talc. The season of collection and the medium used for propagation were the factors of main importance. Phytohormone treatment failed to demonstrate appreciable effect, no difference in rooting response could be attributed to the kind of sand used, but there were indications that response increased with the amount of sedge peat in the medium.

Rooting of white pine cuttings collected in late August and propagated in a sedge peat medium was 62%, earlier and later collections gave substantially less rooting. Sand only and the sphagnum peat media were generally inferior to the sedge type of peat. At the optimum season of collection the sphagnum peat effected 50% rooting.

The late July collection of white spruce cuttings effected rooting of 90% of the plain cuttings when propagation occurred in a sedge peat medium. Low percentages rooted in sand or sphagnum peat media. Cuttings with a heel of old wood tended to be superior to plain cuttings in respect to survival and rooting.

Preliminary experiments with spring and early summer collections of both species resulted in slight rooting. Likewise, greenhouse propagations of dormant material gave very poor results.

An earlier communication dealt briefly with results of experiments on rooting cuttings of white pine (*Pinus strobus* L.) and white spruce (*Picea glauca* (Mœnch)) (6). This article deals with the same experiments in greater detail. The development of efficient methods for the vegetative propagation of economic native conifers is an important phase of a program of tree breeding. The investigations herein reported developed from a series of experiments in which cuttings of Norway spruce (*Picea Abies* (L.) Karst.) were used (2, 5-8, 11-19).

A number of other investigators recently have reported on the vegetative propagation of pines and spruces (1, 3, 4, 9, 10, 20-26, 28-30).

Experimental

The experiments on the propagation of white pine and white spruce cuttings involved consideration of the period of collection, media, type of cutting, and various chemical treatments. Most of the experiments were of factorial design, all were arranged with treatments replicated and groups randomized

¹ Manuscript received February 6, 1942.

Contribution from the Dominion Forest Service, Ottawa, and the Division of Biology and Agriculture, National Research Laboratories. Part of a co-operative project of the Subcommittee on Forest Tree Breeding, Associate Committee on Forestry. N.R.C. No. 1043.

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in the propagating frames (27). Data were subjected to the analysis of variance procedure. Methods of collecting, treating and planting cuttings, the outdoor propagation frames, the various media, and the observations and measurements recorded have been described in earlier articles on the propagation of Norway spruce (7, 8, 17, 18).

The experiments herein described in detail were all initiated during the summer and autumn of 1939 at the Petawawa Forest Experiment Station, Chalk River, Ont. The four experiments dealing with white pine involved the use of 3200 cuttings and the four similar experiments with white spruce, 5480 cuttings. White pine cuttings for Experiments 1 and 2 were taken from trees 10 to 15 yr. of age. Only two branches were collected from any one tree. The terminal 5 to 10 cm. of second order twigs was used for cuttings. Cuttings for Experiments 3 and 4 were taken from the lower part of trees that were 16 yr. of age and somewhat larger than those mentioned above. All white spruce cuttings were taken from the lower part of trees 17 yr. of age. Cuttings were full length and ranged from 5 to 10 cm. in length. Those of Experiments 5 and 6 comprised cuttings with and without a heel of old wood. In Experiments 7 and 8 only full length plain cuttings were used. Experiments 1 to 4 with white pine and 5 to 8 with white spruce were propagated in the same media and frames as Experiments 1 to 4 of a paper dealing with Norway spruce (7).

WHITE PINE

Experiment 1

Bimonthly collections were taken during July and August and planted in three media, namely, sand, sand mixed with one-third by volume of sphagnum peat, and sand with one-third volume of sedge peat. Each collection comprised groups of untreated cuttings and groups treated with powdered talc alone and containing 10, 100, and 1000 p.p.m. (parts of chemical to a million parts of the mixture by weight) of indolylacetic acid.

Experiment 2

The bimonthly collections of Experiment 1 were continued through September and October. Media were reduced to two by eliminating the sphagnum peat mixture.

Experiment 3

One collection of cuttings was taken and planted late October. There were three types of cuttings, namely, Type 1, first order terminal twigs; Type 2, second order terminals, and Type 3, second order laterals. These types have been described and illustrated in an earlier article (8). Cuttings were planted in the two peat media described under Experiment 1.

Experiment 4

Cuttings were collected late October and planted in 28 different media in compartments 1×3.5 ft. in size. The media comprised three grades of sand and various proportions of sedge peat and sphagnum peat, and have already been described in detail (7).

WHITE SPRUCE

Experiments 5 and 6

These experiments were similar to Experiments 1 and 2 in respect to collections, media, and chemical treatments. Both plain cuttings and cuttings with a heel of old wood were used.

Experiment 7

Six types of cuttings were planted in the two peat media described under Experiment 1. These six types of cuttings already have been described in an experiment dealing with Norway spruce (8). The experiment comprised one late October collection of cuttings.

Experiment 8

A late October collection of full length plain cuttings was planted in the 28 media used for Experiment 4 (7).

Cuttings of all these experiments were removed for counts and measurements in September, 1940.

A number of earlier experiments with both white pine and white spruce were initiated during 1938 and the spring of 1939. These experiments dealt with both greenhouse and outdoor plantings in sand and sand with sphagnum peat. Results of these experiments will not be given in detail but will be briefly summarized.

Results

White Pine

Table I indicates the effect of season of collection and propagation medium on the responses of white pine cuttings of Experiments 1 and 2. Since the phytohormone treatment failed to have any significant effect, the data are averages over all treatments with indolylacetic acid, excepting the 1000 p.p.m. level in Experiment 1 which was somewhat injurious. In the sedge peat medium survival and rooting were low for the mid-July collection but increased rapidly. The best rooting, 62%, was obtained with the late August collection. Thereafter a general decline in rooting was noted. However, survival was maintained and development of new growth was somewhat greater. During September and October rooting averaged a little better than 40%. The development of new growth and the length of roots was substantially greater in the sedge peat medium than in sand. Most of the cuttings had only one root, though a few had two or three roots. In sand only there was slight rooting and survival in the first four collections (Experiment 1), hence the data were not included in the table. The four later collections in sand (Experiment 2) indicated substantial survival, but rooting was low, ranging between 12 and 22%. In the sphagnum peat medium survival and rooting were not as good as in sedge peat.

Cuttings of Experiment 3 survived to the extent of about 60% in both media but average rooting was low. Approximately 21% of the cuttings in sphagnum peat rooted, against 10% in the sedge peat medium. Type of cuttings had no appreciable effect on rooting in sedge peat. In the sphagnum



1



2



3

FIG. 1. Miscellaneous group of white pine cuttings showing size and character of roots, callus and new growth.

FIG. 2. Unit of 10 white spruce cuttings with a heel of old wood propagated in sedge peat medium.

FIG. 3. Surviving cuttings of unit of 10 plain white spruce cuttings (without a heel of old wood) propagated in sedge peat medium.

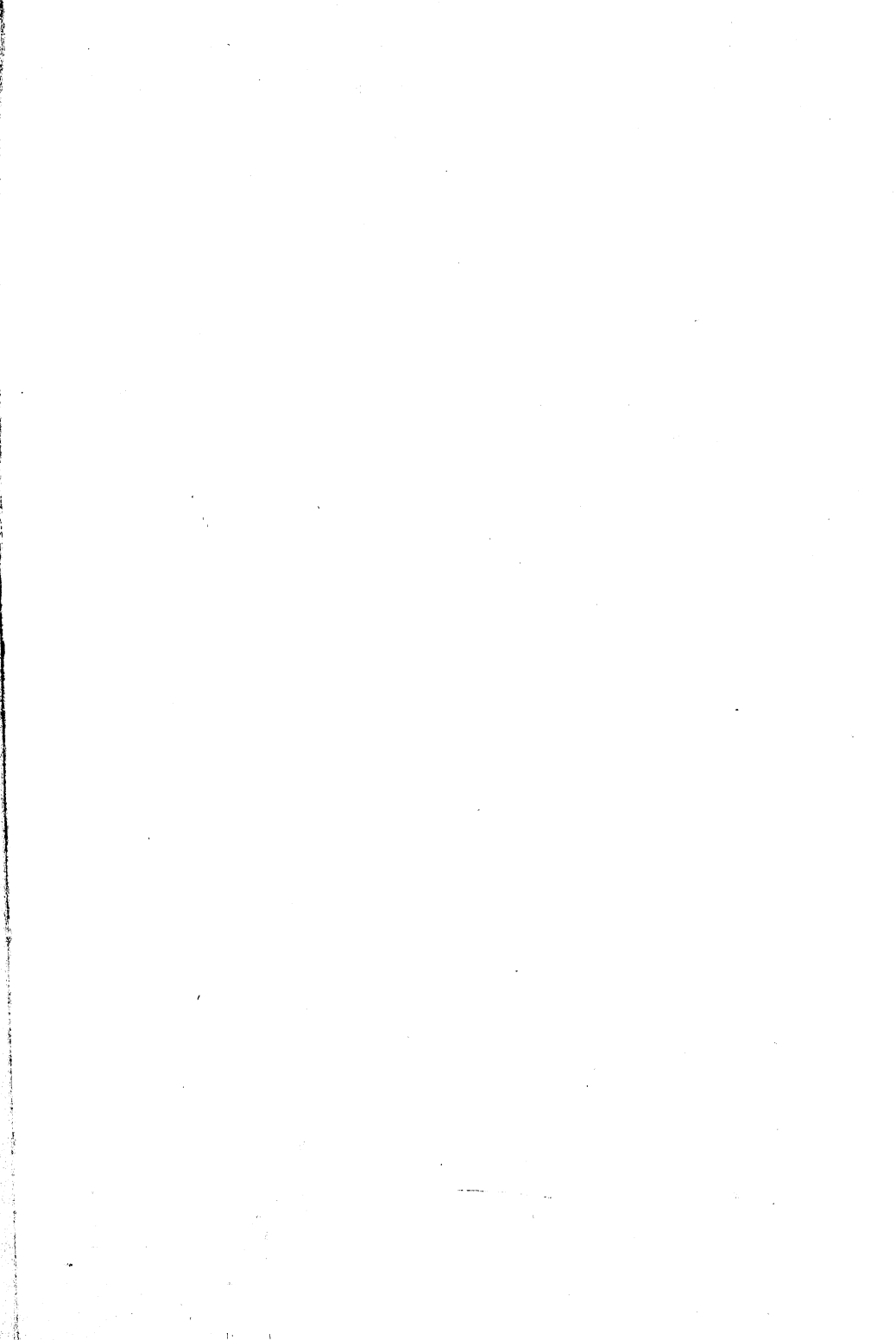


TABLE I
ROOTING RESPONSES OF WHITE PINE CUTTINGS

	Date of collection															
	Experiment 1					Experiment 2										
	July 18	Aug. 1		Aug. 15		Aug. 29		Sept. 18		Oct. 2		Oct. 16		Oct. 30		
	Sedge peat	Sedge peat	Sphagnum peat	Sedge peat	Sphagnum peat	Sedge peat	Sphagnum peat	Sedge peat	Sand peat	Sedge peat	Sand peat	Sedge peat	Sand peat	Sedge peat	Sand peat	
Number of surviving cuttings, %	25	0	57	37	100	85	92	47	80	51	86	66	85	68	89	88
Number of rooted cuttings, %	22	0	32	10	57	50	62	7	44	12	47	19	44	14	36	22
Number of cuttings rooted as percentage of those surviving	90	—	56	27	57	59	68	16	55	24	55	29	52	21	41	25
Number of roots per rooted cutting	1.1	—	1.0	1.5	1.3	1.6	1.2	1.0	1.2	1.8	1.2	1.5	1.3	1.1	1.2	1.3
Length of roots per cutting rooted, mm.	•								146	93	149	86	157	50	162	96
Mean root length, mm.	•								119	51	123	58	123	43	129	76
Number of cuttings with new growth, %	17	0	30	2	40	26	57	0	60	4	67	22	64	16	80	43
Number of cuttings with new growth as a percentage of those surviving	70	—	52	67	40	24	62	—	75	8	78	33	75	24	90	49
Number of rooted cuttings with new growth as percentage of those rooted	67	—	61	25	43	30	76	—	77	25	79	53	76	36	100	59
Number of rooted cuttings with new growth as a percentage of those with new growth	86	—	67	100	81	75	83	—	57	75	55	45	48	31	45	30

* Many of the roots were broken on removal and therefore data for lengths are not given.

TABLE II
ROOTING RESPONSES OF WHITE SPRUCE CUTTINGS IN A SEDGE PEAT MEDIUM

	Kind of cutting	Date of collection											
		Experiment 5						Experiment 6					
		July 13	July 27	Aug. 10	Aug. 24	Sept. 16	Sept. 30	Oct. 14	Oct. 28	Oct. 14	Oct. 28	Oct. 14	Oct. 28
Number of surviving cuttings, %	Plain Heels	46 94	94 92	56 76	56 76	24 44	45 26	26 44	24 21	26 44	24 21	26 44	24 21
Number of rooted cuttings, %	Plain Heels	38 76	90 84	44 62	32 56	16 28	37 21	23 23	15 14	23 23	15 14	23 23	15 14
Number of cuttings rooted as a percentage of those surviving	Plain Heels	83 81	96 91	79 82	57 74	67 64	82 81	88 52	63 67	88 52	63 67	88 52	63 67
Number of roots per rooted cutting	Plain Heels	2.9 2.9	3.4 3.9	3.6 3.3	3.1 2.7	2.0 2.5	2.6 2.2	1.9 2.0	2.1 2.0	1.9 2.0	2.1 2.0	1.9 2.0	2.1 2.0
Length of roots per cutting rooted, mm.	Plain Heels	259 254	233 492	425 347	242 253	94 205	183 119	128 94	128 139	128 94	128 139	128 94	128 139
Mean root length, mm.	Plain Heels	91 87	68 127	117 104	79 94	47 81	71 54	68 48	60 69	68 48	60 69	68 48	60 69
Number of cuttings with new growth, %	Plain Heels	10 8	14 2	6 2	16 6	2 5	10 7	4 7	8 6	4 7	8 6	4 7	8 6

peat 15% of the cuttings of Types 1 and 3 rooted, against 33% of Type 2. Cuttings of Type 1 developed more new growth than those of Types 2 and 3. The respective percentages were 53, 32, and 27%, averaged over both media. Whereas 44% of the cuttings planted in sedge peat had new growth, the value was 31% for sphagnum peat plantings.

Survival and rooting of cuttings of Experiment 4 were not affected significantly by the texture of the sand nor by the proportion of peat. However, there were differences between the average values for sand only and the two different types of peat. In sand, survival was 54% and rooting, 5%; in sedge peat the values were, respectively, 71 and 16%; and in sphagnum peat, 65 and 15%. New growth was developed to the extent of 15% in sand, 50% in sedge peat, and 31% in sphagnum peat. Fig. 1 shows a miscellaneous group of cuttings from this experiment, depicting size and character of root, callus formation, and new growth.

None of the authors' earlier experiments with white pine resulted in appreciable rooting. While results in the greenhouse and outdoors in sand were negative, a midsummer collection in 1938 planted in sphagnum peat and sand resulted in about 4% rooting. Greenhouse experiments with summer collections of cuttings propagated in sand and sedge peat and sand using bottom heat (25) speedily resulted in 100% mortality.

White Spruce

All cuttings of Experiment 5 planted in sand died, and less than 1% of those of Experiment 6 were rooted. Sphagnum peat plantings in Experiment 5 resulted in approximately 2% rooted cuttings. In consequence the data given in Table II are limited to the results from cuttings planted in the sedge peat medium. The data are averages over all indolylacetic acid treatments. Date of collection and type of cuttings both had a marked bearing on the responses. Rooting reached a peak of 84 to 90% in the late July collection. Succeeding collections indicated a gradually deteriorating response. Cuttings with a heel were, on the average, substantially better than plain cuttings. When rooting was at its peak, however, plain cuttings rooted fully as well or better than those with a heel. Length of root attained a maximum of 492 mm. per rooted cutting with a heel for the late July collection, and 425 mm. for plain cuttings of the mid-August collection. Root length declined markedly in later collections. New growth development was rather poor, and plain cuttings appeared superior to those with a heel in this respect. Groups of white spruce cuttings from the July 27 collection, plain and with a heel of old wood, are shown in Figs. 2 and 3.

None of the indolylacetic acid treatments showed a significant effect on rooting or survival. In Experiment 5 indolylacetic acid affected the number of roots per rooted cutting with new growth. The average values were 2.5, 2.7, 3.1, 3.1, and 3.5 roots, respectively, for untreated, talc treated, and treated with 10, 100, and 1000 p.p.m. of indolylacetic acid in talc; the necessary difference for the 5% level of significance was 0.16.

There was 100% mortality of the cuttings of Experiment 7 planted in sphagnum peat. Survival and rooting in sedge peat were poor and it was impossible to demonstrate significant variation in the responses of the six types of cuttings, though certain differences were suggested. Survival of first order terminal cuttings (Type 1) was 5% and rooting, 3%, whereas the corresponding average values for the other five types were 22 and 8%. The best rooting, 16%, was shown by second order terminal cuttings (Type 2). Survival of shortened first order terminal cuttings (Type 6) was about 25% with 3% rooting.

Survival and rooting of cuttings of Experiment 8 were low for plantings in the sedge peat medium, whereas there was 100% mortality of cuttings planted in sand or sphagnum peat. Rooting of 12% occurred in the medium containing equal volumes of sedge peat and sand. The response was poorer for media with a lower proportion of sedge peat.

The undesignated experiments with white spruce resulted in very few rooted cuttings. Early survival counts indicated that cuttings from the lower part of the tree survived in greater percentages than those from the upper part and that lateral cuttings were superior to terminal in this respect (11). The percentage of rooted cuttings from early spring plantings in sand was negligible. Likewise, greenhouse propagation of dormant material taken throughout the winter has proven unsatisfactory; in several such experiments rooting was of the order of 10% and less.

Discussion

The results indicate that cuttings of white pine and white spruce can be rooted in substantial percentages under outside conditions. Period of collection and propagation medium appear to be the limiting factors. The optimum time of collection for white pine was mid- to late August, that for white spruce late July. Cuttings of both species taken before the optimum period showed low survival, which may be related to inadequate lignification. Collections of white pine taken later than the optimum period maintained a high percentage survival and it is possible that substantially higher rooting might have been obtained had the cuttings been left in the frames for a second season. In contrast to the white pine, white spruce cuttings taken after the optimum period demonstrated markedly poorer survival. The sedge type of peat medium gave the best results with both species; indeed it was an absolute necessity for any success with white spruce. White pine, on the other hand, did root to an appreciable extent in sand and sand mixed with sphagnum peat. The beneficial effect of sedge peat may be related to available nutrient factors (16). The results indicate that cuttings of these species root much better under outside conditions than in the greenhouse.

The data indicate that the latter half of August was the optimum period for collecting white pine cuttings. At that season the average rooting of cuttings propagated in sedge peat medium was about 60%. These cuttings were from trees 10 to 15 yr. of age. Cuttings of Experiments 3 and 4 were

from 16-yr. old trees which may account for the somewhat poorer rooting, since it is known that cuttings from older trees do not root so readily (30). Furthermore, it should be noted that these experiments were conducted at an unfavourable season, hence the relative responses of the various types of cuttings and media would not necessarily hold at the optimum season. This observation also relates to Experiments 7 and 8 with white spruce.

Rooting of white spruce cuttings was between 80 and 90% when taken late in July and planted in a sedge peat medium. While rooting of white spruce under optimum conditions approximates the high percentages readily achieved with Norway spruce, satisfactory results with the latter species may be obtained in collections taken over a much longer period. Further, the presence of a heel of old wood is generally favourable to white spruce but usually injurious with Norway spruce. Another striking difference relates to the superior development of new growth by Norway spruce cuttings (7, 8).

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THE EFFECTS OF AMOUNT AND DISTRIBUTION OF RAINFALL ON THE PROTEIN CONTENT OF WESTERN CANADIAN WHEAT¹

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Abstract

Data for 14 yr. were used to determine the average effect of rainfall on the protein content of wheat grown at seven stations in the dry belt of southwestern Saskatchewan. Employing the concept of a regression integral, and working with the rainfall for consecutive five-day intervals from April 1 to August 3, a curve was obtained which represents the change occurring during the growing season in the average regression coefficient (i.e., the average unit decrease in protein content per unit increase in rainfall). It was found that 34% of the residual variance for protein content can be ascribed to variations in rainfall. Above average rainfall during the growing season generally tends to reduce protein content, but this tendency is much more marked during April and early May, and during the latter part of July. These are also the periods during which average rainfall is lowest. The effect on protein content of precipitation occurring during the previous fall is of minor importance.

The study of the effects of weather on the yield and quality of cereal grains is as old as agricultural science itself and a voluminous literature on this subject has gradually accumulated (1, 5). Additional investigation has been stimulated in recent years by the development of advanced statistical methods which make possible a more objective, precise, and quantitative interpretation of the data. Although statistical studies have not yet achieved the goal of accurate prediction of yield and quality from meteorological data, a great deal has been learned of the nature and magnitude of the relationships involved. However, there is still need for additional information, and the present paper represents an attempt to obtain further quantitative data on the effects of variations in the amounts and distribution of seasonal rainfall on the protein content of wheat grown under dry-farming conditions.

Observational Data

The annual protein surveys of this laboratory have made available detailed data on the protein content of wheat grown in Western Canada during each of the past 14 yr. In the area covered by the survey there are a number of meteorological stations many of which have recorded daily precipitation data over the whole of this period.

From the two sets of data thus accumulated, it was necessary to select suitable series for the present investigation. As a first step, it was decided to limit the study to a comparatively small area, in one soil zone, in which

¹ Manuscript received January 17, 1942.
Contribution from the Grain Research Laboratory, Board of Grain Commissioners for Canada, Winnipeg, Man. Published as paper No. 193 of the Associate Committee on Grain Research.

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gross differences in climate do not exist. The brown soils area in southwestern Saskatchewan was finally selected, not only because it met the above criteria, but also because it represented an area of low precipitation where variations from the average might be expected to have marked effects.

Examination of the Monthly Records of the Meteorological Service of Canada showed that it was possible to select, in the prescribed area, a total of six stations (Table I) having complete daily precipitation records for the

TABLE I

RAINFALL DISTRIBUTION COEFFICIENTS AND PROTEIN LEVELS FOR EACH STATION AND EACH YEAR

Station	Year	Rainfall distribution coefficients (in 0.01 in.)							Protein level, % P
		ρ'_0	ρ'_1	ρ'_2	ρ'_3	ρ'_4	ρ'_5	z	
Swift Current	1927	49.28	-1.02	-6.90	0.91	0.08	-3.70	525	11.1
	1928	34.92	11.32	-2.23	-3.35	2.27	3.90	580	13.1
	1929	29.36	2.80	-5.96	-3.88	1.54	0.13	112	15.1
	1930	27.92	1.05	-5.15	-1.97	-1.39	2.18	221	15.2
	1931	19.60	6.80	-0.53	-1.74	-0.49	1.31	528	15.1
	1932	43.44	11.67	-2.00	-3.55	-1.93	1.39	467	14.6
	1933	27.24	-0.38	-4.48	-2.33	0.57	0.08	480	15.2
	1934	28.84	7.63	-5.24	-2.56	1.71	0.36	739	15.2
	1935	40.48	9.26	-0.21	-0.08	-0.72	2.09	263	14.7
	1936	19.96	0.95	-4.39	-0.57	-0.35	0.32	276	15.4
	1937	12.12	3.49	0.46	0.47	1.23	-0.20	290	16.2
	1938	21.08	-0.37	-4.23	1.07	0.31	0.06	250	14.0
	1939	45.88	5.80	-9.16	-2.33	3.67	0.20	407	13.2
	1940	28.28	2.15	1.11	-1.77	5.09	-0.01	155	12.8
Regina	1927	42.76	8.20	-1.99	-4.93	-1.02	-2.04	413	11.9
	1928	38.56	5.50	-7.13	-5.17	2.15	3.12	689	13.0
	1929	17.32	-0.98	-3.67	-1.41	1.94	0.31	107	13.9
	1930	21.84	0.12	-3.51	-0.19	-0.03	0.63	217	14.0
	1931	20.32	9.16	-0.45	-3.11	-1.84	0.12	285	14.8
	1932	28.20	3.00	-5.95	-2.02	0.80	1.52	380	15.6
	1933	35.20	-2.51	-7.99	-0.87	4.21	-2.57	380	14.7
	1934	22.48	5.59	-2.51	-1.36	0.97	-0.26	467	14.7
	1935	45.36	5.83	-5.03	2.72	3.88	2.04	335	13.9
	1936	25.36	-0.26	-6.31	-0.21	0.09	0.75	434	15.0
	1937	19.28	4.54	-1.83	-0.05	1.29	-0.10	224	15.6
	1938	25.92	2.51	-3.79	-0.77	-1.50	0.18	252	14.9
	1939	32.64	7.54	-7.16	-5.76	0.80	2.37	265	15.5
	1940	22.44	3.94	0.96	-1.49	2.24	-0.58	253	15.1
Moose Jaw	1927	31.08	1.35	-4.86	0.51	0.65	-2.37	526	11.5
	1928	36.36	5.70	-8.26	-6.07	2.13	2.82	355	12.9
	1929	14.16	-0.40	-3.59	-0.64	1.22	-0.08	162	15.2
	1930	21.56	1.27	-1.20	0.43	-0.52	0.33	228	15.0
	1931	21.96	7.66	-3.18	-3.40	0.21	1.98	385	14.6
	1932	39.80	5.92	-9.42	-4.75	1.37	2.24	531	14.5
	1933	37.92	-2.13	-8.91	-0.74	3.10	-0.96	451	14.9
	1934	23.12	4.86	-4.79	-1.70	1.74	0.26	533	14.7
	1935	44.64	5.74	-0.71	6.63	1.19	0.09	286	12.8
	1936	30.04	-1.45	-6.43	2.18	-0.76	-0.97	412	14.6
	1937	19.96	6.47	1.99	-0.21	-0.26	-2.36	145	14.9
	1938	26.96	0.49	-3.91	-0.22	-2.24	0.72	231	13.9
	1939	32.36	3.46	-9.15	-3.11	3.34	2.30	279	14.2
	1940	29.24	4.03	0.13	-1.73	3.88	-0.86	136	14.4

TABLE I—*Concluded*RAINFALL DISTRIBUTION COEFFICIENTS AND PROTEIN LEVELS FOR EACH STATION AND EACH YEAR
—*Concluded*

Station	Year	Rainfall distribution coefficients (in 0.01 in.)							Protein level, % P
		ρ'_0	ρ'_1	ρ'_2	ρ'_3	ρ'_4	ρ'_5	s	
Beechy	1927	37.44	1.46	-2.94	0.08	-0.50	-1.91	325	12.0
	1928	28.16	3.71	-2.35	-3.25	1.24	2.46	629	12.7
	1929	19.76	-0.76	-4.32	-1.56	0.92	1.26	172	15.0
	1930	23.80	3.94	-2.17	-1.05	-1.39	-0.40	207	15.6
	1931	17.52	6.70	0.93	0.06	0.40	2.52	433	15.3
	1932	28.60	6.33	-2.28	-3.00	-0.92	0.86	444	15.3
	1933	19.28	-1.49	-2.71	-0.76	1.15	-1.06	366	15.7
	1934	17.64	2.91	-5.46	-1.63	2.45	0.59	486	16.4
	1935	40.68	8.68	-2.12	-2.32	-3.03	0.87	159	15.6
	1936	24.16	2.96	-4.18	-0.09	-0.51	-0.97	328	15.9
	1937	28.24	7.94	0.58	0.74	0.09	-2.51	267	15.9
	1938	20.60	2.01	-2.62	0.79	-0.39	0.06	205	15.4
	1939	43.80	9.47	-10.48	-5.67	2.36	1.83	395	14.3
	1940	24.00	6.21	1.04	-0.56	1.46	-0.54	136	15.0
Assiniboia	1927	22.36	0.73	-4.46	0.49	0.70	-1.33	507	12.8
	1928	38.28	8.71	-4.07	-7.12	1.02	2.12	252	13.3
	1929	6.76	-0.06	-1.48	-0.47	0.32	0.00	132	15.8
	1930	20.52	-0.44	-2.45	-0.31	-0.23	0.65	235	16.4
	1931	24.60	10.66	0.27	-1.56	0.78	2.23	271	15.6
	1932	29.44	2.75	-7.92	-3.41	1.60	2.50	416	16.6
	1933	30.68	-0.73	-6.56	-1.68	1.35	-0.32	363	16.0
	1934	22.92	3.48	-5.71	-1.17	1.91	-0.01	631	15.6
	1935	48.16	5.79	-7.26	-1.21	0.49	1.23	225	15.3
	1936	16.84	2.56	-1.28	0.65	-0.95	-0.34	289	16.5
	1937	19.56	8.33	3.25	-1.50	-0.35	-0.92	211	16.4
	1938	41.28	4.61	-6.55	-2.16	-3.04	0.51	403	14.7
	1939	46.24	3.43	-12.70	-3.55	3.51	1.67	381	14.4
	1940	41.40	5.44	-0.07	0.00	4.78	-0.48	153	13.7
Anglia	1927	42.84	1.40	-5.45	1.24	-1.31	-2.54	195	11.6
	1928	32.28	9.75	-0.81	-1.75	1.41	2.21	468	13.4
	1929	29.68	1.24	-3.86	-4.99	0.96	1.27	314	13.7
	1930	25.60	5.34	2.20	-0.47	-2.24	-0.05	160	13.7
	1931	11.96	4.28	0.35	-2.12	-1.22	0.64	437	14.4
	1932	17.36	3.33	-1.78	-2.01	-1.42	0.17	285	14.4
	1933	11.28	-0.26	-1.55	-0.22	-0.35	-0.57	226	14.8
	1934	12.60	3.05	-2.59	-2.84	-0.14	0.99	318	15.7
	1935	24.92	2.36	0.56	-1.84	-1.23	1.58	99	14.8
	1936	10.40	-1.06	-1.51	-0.31	-0.19	0.74	185	15.6
	1937	18.40	6.15	-0.33	-1.94	-1.05	-1.45	146	15.1
	1938	29.44	0.78	-2.30	1.64	-0.04	1.33	220	13.9
	1939	30.36	3.94	-8.95	-4.89	2.67	2.68	477	14.3
	1940	35.40	7.33	-0.41	-4.12	-0.24	-0.42	184	13.7
Alsask	1927	62.72	3.98	-5.02	-4.10	-3.12	-2.04	398	11.0
	1928	30.48	2.26	0.32	-2.86	1.39	0.92	386	13.4
	1929	19.84	-1.59	0.54	-3.03	1.08	-2.31	288	15.2
	1930	40.36	6.63	-0.95	-0.12	-1.88	3.50	108	14.2
	1931	13.88	4.26	0.19	-1.79	-1.56	-0.16	602	14.7
	1932	37.60	-0.52	-2.09	-3.04	0.64	-1.34	288	14.5
	1933	14.44	0.21	-1.85	-0.26	-0.99	-0.54	347	15.0
	1934	21.48	0.78	-4.73	0.19	0.59	0.52	393	15.9
	1935	21.48	-0.38	-0.99	-0.61	-0.57	1.10	205	16.3
	1936	18.96	1.64	-2.61	-0.35	-0.79	0.01	291	16.8
	1937	16.68	4.34	-0.41	-1.03	0.22	-0.59	316	15.5
	1938	32.56	5.70	-1.64	-0.76	-1.54	1.46	218	15.2
	1939	32.56	2.83	-8.11	-2.90	2.54	-0.32	303	15.4

14 yr. period. A seventh station, Alsask, was also added, though daily records were not complete for 1940.

The protein level for wheat grown around each meteorological station was taken as the mean of results for all samples taken from carlots shipped from points within a radius of 15 miles of the station. In so far as precipitation records taken at a central point may be considered representative of such an area, this procedure seemed desirable since it tends to reduce sampling error and thus to produce a more reliable series of values. The annual protein levels for each station are given in the last column of Table I. Each figure represents the mean of results obtained for an average of 19 samples: the lowest number of samples represented is one, and the highest is 40.

The study of the effects on protein content of amount and distribution of precipitation was confined to the period represented by the months of April, May, June, July, and the first three days of August. For purposes of calculation this period was divided into 25 intervals of five days each.

Account was also taken of the precipitation occurring during the three months, August, September, and October, of the fall preceding the growing season under study. However, no attempt was made to study the effect of distribution of rainfall during this period, the total amount of rainfall for the three months was treated simply as a single additional rainfall variant.

The practice of considering the precipitation affecting the crop as that occurring during the growing season and during three months of the previous fall, and of disregarding winter precipitation, is now generally adopted in Western Canada. The practice stems from the investigations of Barnes and Hopkins (2) who state, in a study of crop production at Swift Current, that "no appreciable increase in soil moisture has been observed from the presence of snow." In Western Canada precipitation during the winter months occurs as snow, which falls on frozen ground and cannot be absorbed. An appreciable proportion of the snow is lost by evaporation and most of the remainder is lost in the spring run-off because the snow melts before the underlying ground can thaw.

Methods of Analysis

Fisher's concept (4) of a regression integral, involving a regression function varying continuously with time, was employed in this investigation. Details of the practical application of this concept to the study of the effect of rainfall on the yield of Rothamsted plots are given in full by Fisher (4), together with a thorough discussion of the advantages and disadvantages of the method. Hopkins (7) also employed this method in his study of the effect of rainfall on the yield of wheat at certain stations in Western Canada. He worked with data for eight stations, 10 years, and a 125 day period divided into 25 five-day intervals, thus providing a pattern for the present study. In these circumstances the method need not be fully described in this paper.

In this study, protein content at any station is taken as a function of the amount and distribution of rainfall over the growing season and the amount of rainfall for the previous fall. The regression integral therefore takes the form

$$P = c + \int_0^T a(t)r(t)dt + kz$$

where $a(t)$ represents the effect on protein content of a unit increment of rain falling at time t , and $r(t)dt$ the increment of rain falling in the time interval dt ; the integral is taken over the 125 days of the growing season. The last term takes account of the previous fall rain, z representing the amount and k its regression coefficient. The quantities to be evaluated from the available data are $a(t)$ and k .

Following Fisher's method, the seasonal rainfall, $r(t)$, was expressed as a function of time in terms of orthogonal polynomials of the fifth degree, there being one equation for each station in each year. Each equation is fitted (13, pp. 279-289) to the appropriate set of 25 data representing rainfall for five-day intervals during the growing season. The six coefficients, ρ'_0 to ρ'_5 (corresponding to Fisher's coefficients a' to f'), of each equation were then available as independent rainfall variables with which to correlate the protein data, P . As pointed out previously, there was also available a seventh set of rainfall variables, z , representing the precipitation of the previous fall. Values of ρ' , z , and P , for each station in each year, are listed in Table I.

The correlations were based on the residuals after removing the variation due to stations (six degrees of freedom) and years (13 degrees of freedom). This procedure is adopted for several reasons. Although the seven stations selected are in a limited area, it is not valid to assume that the only factors affecting protein content are seasonal variations in rainfall and factors closely associated with it. The annual protein values for each station show differences from year to year which are not ascribable to changes in rainfall. These might arise from differences in dates of seeding, or in the soils, percentages of summer-fallow, etc., represented by the protein samples. Again, in the area represented one might expect a certain correlation to exist between the weather at different stations, and if some extraneous source of variation is common to several stations, a spurious effect will be introduced into the regression. Such interannual heterogeneity is partially eliminated by working with residuals which should represent a homogeneous group of data whose variations arise primarily from the variations that exist within the particular year from which they are drawn. Correlations of these data thus serve to illustrate the effect on the average protein content of the wheat of deviations from the average amount and average distribution of the rainfall.

Fifth degree polynomial functions were fitted to the ρ' , z , and P values for each station giving rise to sets of six coefficients, x_0 to x_5 , describing the inter-annual trends in the data. The values for x , together with the calculated

standard residues, are listed in Table II. Fisher (4), working with continuous series of 60 yr. used x values such as these for eliminating the heterogeneous variation occurring between years. However, in the present investigation, since the values from several stations in a relatively small area are to be posted, it was thought best to remove the interannual variations by adjusting the annual averages over the seven stations. The x coefficients were used simply to examine the interannual tendencies of rainfall and protein over the 14 yr.

TABLE II
ANALYSIS OF INTERANNUAL TRENDS OF RAINFALL AND PROTEIN

Station		ρ'_0	ρ'_1	ρ'_2	ρ'_3	ρ'_4	ρ'_5	z	P^\dagger
Swift Current	Mean	30.600	4.368	-3.494	-1.549	0.828	0.579	378.1	43.500
	x_1	-11.372	-1.970	2.256	1.791	3.092	-0.417	-218.8	7.326
	x_2	13.372	-4.186	-1.785	0.835	4.348	-2.192	-127.2	-40.991
	x_3	-2.122	2.847	2.149	-4.092	1.394	2.454	-126.0	5.888
	x_4	14.519	0.287	1.899	0.262	0.387	-2.190	253.8	-16.392
	x_5	-9.662	2.661	4.222	-1.046	1.966	2.357	-177.2	9.358
	Standard residue	10.849	5.153	3.236	1.298	1.336	1.412	177.3	5.838
Regina	Mean	28.406	3.727	-4.026	-1.759	0.99 ^c	0.392	335.8	44.714
	x_1	-6.824	-0.036	0.916	2.426	0.771	0.380	-155.6	28.244
	x_2	5.392	4.147	2.854	-4.978	-1.133	-0.087	7.6	-15.492
	x_3	-14.786	-2.371	2.015	-0.487	0.760	0.419	-163.6	11.455
	x_4	12.522	2.370	1.873	0.216	1.762	-2.720	165.3	-5.746
	x_5	-1.196	-3.590	0.285	3.264	3.568	1.335	117.3	-6.512
	Standard residue	8.725	3.976	3.119	1.869	1.753	1.675	145.7	4.854
Moose Jaw	Mean	29.226	3.069	-4.449	-0.916	1.075	0.224	332.9	41.500
	x_1	2.526	0.514	2.935	2.116	0.638	-0.587	-211.6	11.370
	x_2	-2.780	-0.584	1.061	-2.588	1.587	-0.614	-166.5	-18.198
	x_3	-5.554	1.603	0.124	-4.882	2.613	2.617	-131.9	20.630
	x_4	14.426	0.783	-1.115	1.346	3.047	-1.886	290.1	-11.868
	x_5	-0.645	-0.088	4.269	2.930	0.943	-1.065	-164.7	1.689
	Standard residue	9.376	3.995	4.270	3.154	1.520	1.722	92.6	7.087
Beechy	Mean	26.691	4.291	-2.791	-1.301	0.238	0.219	325.1	50.071
	x_1	3.440	5.662	-1.026	-0.028	0.888	-1.010	-184.8	22.177
	x_2	10.762	0.222	-0.474	-0.485	1.510	-0.351	-69.5	-33.838
	x_3	-11.677	0.834	2.048	-1.472	1.381	2.457	36.8	9.521
	x_4	2.548	-0.549	1.792	0.422	0.138	-1.488	37.4	-3.277
	x_5	-4.654	-1.168	2.248	0.261	-0.143	0.059	-71.5	5.244
	Standard residue	9.001	3.789	3.500	2.208	1.746	1.643	166.7	5.306
Assiniboia	Mean	29.217	3.947	-4.071	-1.643	0.849	0.536	319.2	52.214
	x_1	21.502	2.632	-1.608	0.912	1.257	-0.552	-37.4	4.674
	x_2	9.534	0.394	0.576	-0.381	2.180	-1.051	-76.0	-38.100
	x_3	0.947	-0.280	1.256	-0.552	3.430	1.791	-192.7	7.203
	x_4	7.372	-1.940	-1.730	2.218	3.613	-1.134	201.8	-10.348
	x_5	-4.494	1.768	6.190	0.225	0.392	-0.883	-248.1	-0.396
	Standard residue	12.906	4.335	4.669	2.421	1.351	1.277	115.2	7.104

† Protein coded: $P = 10$ (percentage of protein - 10).

TABLE II—*Concluded*ANALYSIS OF INTERANNUAL TRENDS OF RAINFALL AND PROTEIN—*Concluded*

Station		ρ'_0	ρ'_1	ρ'_2	ρ'_3	ρ'_4	ρ'_5	z	P^\dagger
Anglia	Mean	23.751	3.402	-1.888	-1.759	-0.314	0.470	265.3	42.214
	x_1	-5.112	-0.384	-1.028	-1.588	1.095	0.986	-90.0	18.862
	x_2	32.876	4.156	-4.192	-0.824	1.718	-0.688	68.5	-28.909
	x_3	-2.417	3.938	2.633	-3.896	0.002	0.963	129.2	-0.083
	x_4	-1.450	-1.386	0.124	0.601	-1.507	-2.351	-114.8	-1.740
	x_5	0.173	2.750	4.092	-2.014	0.549	0.990	-98.4	8.736
	Standard residue	5.483	3.078	2.705	1.971	1.371	1.458	133.8	4.116
Alsaask	Mean	27.926	2.138	-2.104	-1.589	-0.307	0.016	318.7	48.538
	x_1	-18.407	0.821	-3.122	2.268	1.562	1.024	-106.6	36.321
	x_2	27.764	3.496	-3.638	-3.015	0.463	-0.900	-11.2	-25.344
	x_3	-5.449	0.793	0.159	-0.758	2.173	0.847	-13.3	3.763
	x_4	15.124	-1.858	-6.262	-1.033	-0.215	-1.492	121.2	-13.362
	x_5	-13.163	-5.023	-1.118	0.176	3.476	0.262	-41.4	16.552
	Standard residue	10.010	2.286	1.315	1.105	1.173	1.848	141.3	4.158

† Protein coded: $P = 10$ (Percentage of protein - 10).

Finally, the regression function $a(t)$ was expressed in terms of orthogonal polynomials of the fifth degree involving the coefficients α_0 to α_5 . The equation then takes the form

$$P = c + \alpha_0\rho_0 + \alpha_1\rho_1 + \alpha_2\rho_2 + \alpha_3\rho_3 + \alpha_4\rho_4 + \alpha_5\rho_5 + kz.$$

The actual calculations were made with the residuals of ρ' and z , obtaining, by the method of least squares, a set of coefficients α' from which the values of α were derived. These statistics, α values and k , are recorded and discussed in a later section. The function thus evaluated makes it possible to determine the effect on protein content of an additional increment of rainfall occurring at any period during the growing season, or during the three month period of the previous fall.

The method of analysis, based on the concept of a regression integral, has certain advantages over the more commonly used multiple regression method. To divide the season into 25 five-day intervals and obtain the regression on each would entail the solution of determinants of order 26, an undertaking involving an enormous amount of calculation. Moreover, the loss of 26 degrees of freedom would decrease considerably the precision of the test of significance. It should also be noted that the multiple regression method makes no use of the well founded assumption that the regression coefficient of protein content on rainfall is a slow changing function of time.

Two possible objections to the use of the regression integral are worth noting. In the first place, rainfall is not a continuous function of time over the growing season and yet it is so treated. Although there is no reason to believe that this treatment will introduce a bias, it does tend to decrease efficiency by introducing an additional source of variation. Fisher disposes

of the point by suggesting that since the regression function "varies relatively slowly, little would be gained by following, in more detail, the rapid fluctuations of the weather." The second objection relates to the assumption that the effect of rain falling at any time is independent of rain falling previously in the same growing season. Although such a premise may be open to question, it appears reasonably valid for a region in which the soil is seldom, if ever, saturated.

Analysis of Rainfall

The average amount and distribution of seasonal rainfall, for all stations over the 14 yr., are illustrated in Fig. 1. In drawing the graph the average amount of rain falling in each five day interval was plotted against the middle date for the interval. The curve shows that rainfall is normally low in April, rises steadily during May, reaches a peak in June, and decreases during July. This trend is similar to that obtained by Hopkins (9) with rainfall data for 48 yr. at Swift Current, one of the stations included in the present study.

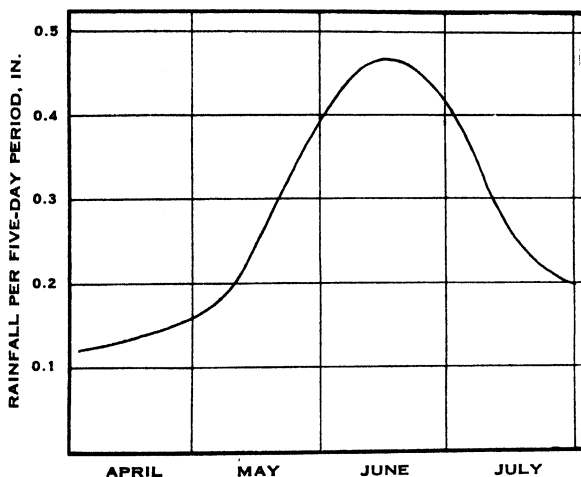


FIG. 1. Average precipitation recorded at seven stations in southwestern Saskatchewan, 1927-1940.

The total average rainfall for the period April 1 to August 3 is seven inches, while the greatest average amount falling in any five day interval is 0.46 in. Such moisture conditions are very close to the minimum necessary for the growth of wheat. If variations in rainfall do affect the protein content of the crop, one would expect to observe a magnification of this effect under such dry conditions.

The characteristics of the seasonal rainfalls at each station are represented by the ρ' coefficients of Table I. There is one line of data for each year at each station. The first column of data, under ρ'_0 , presents the average value of the five-day totals of rainfall for the season. Data in the next column,

ρ'_1 , measure the linear or average rate of increase of this rainfall; ρ'_2 measures the quadratic term in the sequence of rainfall totals; ρ'_3 , the cubic term, and so on. Since these statistics represent only the raw data on which this study was based, no attempt will be made to discuss them in detail.

The results of the analyses of the interannual tendencies of the rainfall data for each of the stations are given in Table II. The x coefficients are similar in nature to the ρ' coefficients described above: x_1 measures the linear trend, x_2 the quadratic trend, and so on. The standard residue is taken as $\left(\sum_{i=1}^{13} x_i^2\right)/8$, assuming that all slow changes have been accounted for by x_1 to x_5 .

In dealing with a short period of 14 yr., one would not expect an analysis of the data to produce conclusive evidence of progressive changes in the amount and distribution of rainfall from year to year. In general, this expectation was borne out in the present study. However, examination of Table II will show that at Alsask and Anglia the magnitude of the quadratic terms (x_2) in the ρ'_0 sequence indicates that a series of dry years occurred between 1931 and 1937. These together with significant x_1 and x_2 values in the Alsask distribution coefficients ρ'_2 and ρ'_3 , suggest the presence of progressive change. It is well known, of course, that a cycle of drought years did occur in southern Saskatchewan between 1931 and 1937, and it seems probable that the significance of this trend could be established by the study of data for a long period of years. However, in the present study, which deals with only 14 yr., interannual differences in rainfall must be considered to arise fortuitously, and it must be concluded that the climatic conditions of the area did not exhibit any marked changes.

Regression of Protein Content on Rainfall

The following values were obtained for the coefficients of the equation representing the regression of protein content on rainfall (see p. 218).

$$\begin{array}{llll} \alpha_0 = -0.1213 & \alpha_1 = 0.0713 & \alpha_2 = -0.0654 & k = -0.00811 \\ \alpha_3 = -0.0497 & \alpha_4 = 0.00881 & \alpha_5 = 0.00001 & \end{array}$$

Protein was measured in units of 0.1% and rainfall in 0.01 in.

The significance of the correlation between protein and the various rainfall coefficients may be determined by analysing the variance of the protein residuals into portions due to the rainfall regression and to deviations from the regression. The results of the analyses are shown in Table III. From the first analysis it is apparent that the correlation coefficient ($R = .632$) is highly significant, the 1% point being .475. Following Fisher's procedure (4), the percentage of the residual variance accounted for by variations in rainfall was calculated and found to be 34%, a high proportion for a single meteorological variant. The remaining 66% is accounted for by environmental factors not associated with rainfall, and by fortuitous experimental errors. These latter may arise from various causes, among which may be mentioned:— differences in seeding times; differences in the soils represented at each station in each year; and differences in the effectiveness of equal

increments of rain falling at each station in each year, it being assumed that a number of light showers are less effective than a steady rain.

More detailed analyses are also presented in Table III. Analysis 2 shows that the major portion of the variation is accounted for by variations in the total seasonal rainfall (ρ_0) although the remaining rainfall variables still have a variance significantly greater than the error. Statistics given in Analysis 3 show that the introduction of fall rainfall does not reduce the variance due to the remaining variables, suggesting that ρ_1 , ρ_2 , and possibly ρ_3 have a greater effect on the protein than z . Analysis 4 illustrates the additional variation accounted for when ρ_1 , ρ_2 , and ρ_3 are introduced, and shows that ρ_4 , ρ_5 , and z are unimportant in the present investigation. It thus appears that a satisfactory correlation can be obtained by considering only the amount of seasonal rainfall along with its distribution as expressed by a cubic function of time.

TABLE III
ANALYSIS OF VARIANCE OF PROTEIN RESIDUALS

Analysis number	Variance due to	Degrees of freedom	Sum of squares	Mean square
1	Rainfall regression $R = .632$	7	1178	168.3**
	Deviations from regression	70	1776	25.4
2	Total seasonal rainfall	1	743.6	743.6**
	Remaining rainfall variants	6	434.4	72.4*
	Deviations from regression	70	1776	25.4
3	Total seasonal rainfall	1	743.6	743.6**
	Fall rainfall	1	31.6	31.6
	Remaining rainfall variants	5	402.8	80.6*
	Deviations from regression	70	1776	25.4
4	Total seasonal rainfall	1	743.6	743.6**
	Linear trend	1	79.6	79.6
	Quadratic trend	1	199.1	199.1**
	Cubic trend	1	113.4	113.4*
	Remaining rainfall variants	3	42.3	14.1
	Deviations from regression	70	1776	25.4

* The variance surpasses the 5% level of significance.

** The variance surpasses the 1% level of significance.

The regression coefficients, given in the first paragraph of this section, make it possible to estimate the average effect on the protein content of the crop of an additional inch of rainfall at any time during the growing season. The difference in the effect of additional rain falling at different periods, i.e., the course of the regression function $a(t)$, is shown by the curve in Fig. 2.

Before considering the curve in detail, it seems best to dispose of the effect of an increase in the total rainfall for the growing season. Returning to the regression coefficients, it will be observed that the comparative magnitude of α_0 indicates that total precipitation has a major effect on protein content.

Decoding the value for α_0 , and expressing the result in simple terms, it may be stated that if the total rainfall is increased one inch above normal, while the rainfall distribution is held constant, protein content tends to decrease by about 0.24%.

The curve in Fig. 2 indicates that additional rainfall generally tends to decrease the percentage of protein in the wheat. It is also clear that the effect of a given increment of additional rainfall depends upon the period during which it falls. In this matter the curve speaks for itself, but summarizing, it may be said that additional rainfall occurring in April, the first half of May, or the last half of July, has an appreciably greater effect on protein content than rain falling during June.

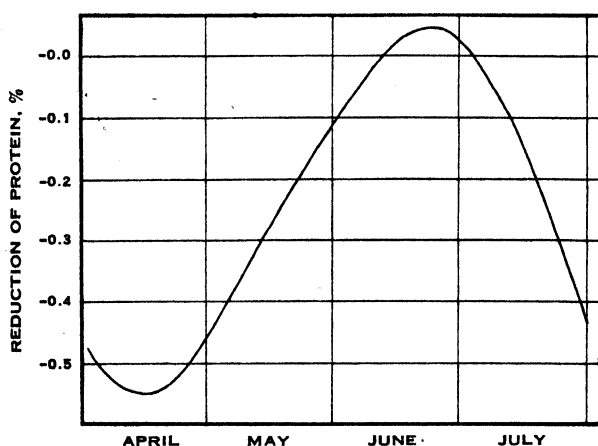


FIG. 2. Average effect of an additional inch of rainfall on the protein content of wheat.

When interpreting the curve, the reader is warned not to generalize too broadly. The curve deals with the average amount and distribution of seasonal rainfall, and the average annual protein level of wheat, at seven stations in the dry area of southwestern Saskatchewan. It illustrates the effect of deviations from the average rainfall in terms of the resulting deviations of the percentage protein from its average value. The curve does not show that each inch of rainfall in early May, for instance, will lower protein content by 0.4%, but only that an increase of an inch over the normal rainfall will result in an average decrease of 0.4% in the normal protein level. The results are actually reported in terms of the effect of an additional inch of rain on percentage protein merely because this is the customary practice. It would be equally correct to report the results in terms of the effect of one inch less rainfall than normal, and perhaps better to report in terms of the effect of a difference in rainfall of from one-half inch below normal to one-half inch above normal. For that matter, it is not necessary, and perhaps even slightly misleading, to select the conventional one inch of rainfall as the reference unit.

However, it will be clear that no change in the regression is involved if it is reported in terms of the effects of one-tenth rather than one inch of additional rainfall.

A further difficulty may arise from the fact that a continuous, smooth curve suggests that the interpretation should be limited to the effect of an additional increment of rain falling at a given time during a very short period. Actually, a consideration of the data on which the curve is based suggests that a detailed interpretation of the curve is not warranted. The additional increment of rain may better be thought of as occurring during a longer period, e.g., the first half of June, when its effects will be represented by the average level of the curve for this period. This procedure seems much sounder, particularly in view of the fact that in the area concerned the average amount of rain falling in June is less than 3 in., and June has normally a higher rainfall than any other month of the growing season.

Returning to the coefficients listed on page 220, it will be noted that the coefficient (k) of the partial regression of protein content on rainfall occurring in the three months of the fall preceding the growing season is -0.0081 . In other words, an inch more rain than normal in the fall decreases the average protein content of the next crop by only 0.08%.

Discussion

Previous statistical investigations of the effect of rainfall on protein content, under semiarid conditions approximating those of the present study, appear to be confined to those of Hopkins (6, 8). He worked with data, collected in certain years during the period 1915 to 1932, for five stations in Western Canada (Lacombe, Lethbridge, Indian Head, Scott, and Rosthern). The average conditions represented in Hopkins' study are similar to those of the present study, as can be seen by comparing the mean total rainfalls for the period May 1 to July 31, which are: Hopkins, 6.4 in.; this study, 6.2 in. It should be noted, however, that there is probably a difference in the average dates of seeding and heading of wheat, those for the present study being somewhat earlier.

Working with the partial regressions of protein content on monthly rainfall totals for May, June, July, and August, Hopkins (6) found that the main effect of rainfall in reducing protein content was exerted during May and June. His data did not justify the conclusion that the amount of rain falling in July or August, or the amount of preseasonal precipitation (August 1 to April 30), modified the nitrogen content significantly. Mean maximum temperature for July or August also failed to show a significant correlation with nitrogen content. However, in a second paper (8), by the use of coefficients designed to weight observed temperatures in proportion to their assumed effect on the respiration of the grain, higher temperatures during the six weeks preceding September 1 were shown to have a moderate positive correlation with protein content.

The results of the present investigation support Hopkins' conclusions concerning the importance of additional rain falling during the early part of the growing season. However, whereas Hopkins found that May rainfall had the greatest effect on protein content, followed closely by June rainfall, in the present study the April rainfall is most effective, May rainfall is moderately effective, and June rainfall is only of minor importance. This difference may be accounted for in part by some difference in the average dates of seeding and of heading represented in the two investigations. Unfortunately, it was not possible to obtain complete data on this point but it seems safe to assume that the average dates of seeding and heading are slightly later for the group of stations examined by Hopkins. It should also be noted that Hopkins did not examine April rainfall which might also have been more effective than May rainfall under the conditions that he studied.

Russell and Bishop (11) in their investigation of the effect of weather on the nitrogen content of barley, also found that rainfall occurring early in the growing season had a major effect on the nitrogen content of the kernels. They suggest that excessive spring rain may reduce nitrogen content by leaching nitrates from the soil. This hypothesis, as Hopkins points out, can hardly apply under the semiarid conditions of the southern and south-central portions of the Prairie Provinces. Under such conditions, Hopkins believes that the main effect is produced in another way, namely, by the stimulation of tillering and general vegetative development produced by early rains. He writes, "as a result of this proliferation, the supply of available nitrogen must be distributed amongst an increased number of culms, and the total leaf area, devoted to the production of carbohydrates, will also be augmented, both circumstances tending towards a diminished proportion of nitrogen in the resulting grain."

This hypothesis appears to require some extension to account for the results illustrated in Fig. 2. Specifically, it seems necessary to provide an explanation for the fact that under dry conditions additional rain occurring in April, prior to seeding, depresses protein content appreciably more than rain occurring when the wheat starts to tiller. On the other hand, it would be unwise to pay too much attention to shape of the curve for April, and particularly to the exact position of the minimum point, since the available data do not serve to establish these with certainty.

The average date of seeding, under the conditions represented in this study, is estimated to be April 30. Fig. 2 shows that additional rainfall has its maximum effect about April 15. It thus seems probable that the mechanism involved at this time is concerned with the condition of the seed bed, and hence with the germination and early growth of the seeds. With rainfall normally very low in April, it appears that above average precipitation may result in an appreciably higher germination than normal and in a heavier stand of more vigorous seedlings. If an average summer then follows, the good start obtained by the crop will result in above normal vegetative growth and thus in a decrease in the protein content of the crop.

It will be observed that the curve in Fig. 2 has a minimum value at about the middle of April, i.e., about two weeks before seeding. Additional rain falling prior to this time appears to have less effect on the protein content of the crop, as does the precipitation occurring in the three months of the preceding fall. Assuming this hypothesis to be correct, some explanation of it may be attempted. On summer fallowed land, such rain will fall on bare ground and may be expected to be less available to the seed for two reasons. In the first place it will gradually penetrate below seeding depth, and in the second place it may be expected to evaporate more rapidly than it would do were the land partially covered as it is as soon as the wheat plants emerge. The first of these reasons applies equally well to stubble land and the second also applies in part if the stubble is worked before seeding. In consequence it does not seem improbable that precipitation occurring during the previous fall and some time before seeding should have less effect on germination and early vegetative growth than rainfall occurring just before seeding time.

In general it appears that in southwestern Saskatchewan rain is available to and used by the growing plants immediately after it falls, but its effect must continue for some time, thus carrying the plants over from one shower to the next. At the beginning of the growing season the young plant, with its short roots, is almost wholly dependent on recent rainfall. As growth continues, and the roots penetrate to greater depths, the plant is able to draw on additional reserves of moisture which have accumulated as a result of earlier rains. Accordingly, it would appear that tillering, which starts early in June, is affected not only by the rain falling during late May, but also by the amount of reserve moisture in the soil. Hence it seems reasonable to assume that rain falling in April may have a fairly direct effect on the vegetative growth occurring more than a month later. It thus appears that the hypothesis put forward by Hopkins (6), concerning the effect of additional rain early in the season on tillering and on the general vegetative growth of the plant, presents a reasonable explanation of the available data.

The curve in Fig. 2 shows that the protein content of the crop is very susceptible to variations from the normal rainfall during the latter part of July. This supports the work of Shutt and Hamilton (12), who concluded, as a result of studies on wheat grown at Scott, Saskatchewan, and at three stations in relatively high rainfall areas, that the "chief environmental factor conducive to high protein content" is "the drying out of soil, due to scanty precipitation and high temperatures, during the latter weeks of development and ripening of the kernel." Many other authors have expressed similar opinions. In his first paper, Hopkins (6) was unable to demonstrate that differences in either rainfall or temperature during the later part of the growing season had any marked effect on protein content. However, as noted above, he did show in his second paper that high temperatures had a moderate effect on protein content. Citing the chemical investigations of McCalla and Newton (10) and of other earlier workers, Hopkins assumes that the effect of high temperature is accounted for principally by increased respiration in

the grain and that it is not necessary to suppose that high temperatures change the rates at which carbohydrates and proteins are translocated to the kernels.

It seems probable that under the dry conditions represented in the present study, deviations from the normal rainfall at the end of July have an effect on the protein content of the grain which is not necessarily associated with any change in air temperature. Under normal conditions, wheat plants grown in southwestern Saskatchewan dry out fairly rapidly during the latter part of the growing season. If the rainfall is below normal at this time, the moisture content of the soil becomes a limiting factor, and it may be assumed that the rate of translocation of both proteins and carbohydrates diminishes rapidly and may even approach zero. If translocation decreases considerably, the effect of respiration in the grain on the subsequent protein content will obviously be of major importance. This mechanism will operate irrespective of air temperatures though it will be of increased importance with higher temperatures. Though it applies only to dry conditions, this hypothesis serves to explain how rainfall in late July may exert an effect on the protein content of the grain which is not directly associated with the possible effects of temperature. On the other hand, since some correlations may be expected between temperature and rainfall, it seems probable that the slope of the curve in Fig. 2 for July represents in part a temperature effect. There is some reason to suppose, however, that the curve for this period represents principally the effect of additional rainfall which maintains the rate, and increases the time, of translocation, and thus offsets the effect of respiration on protein content.

Superficially it might appear that the hypothesis outlined above is not consistent with results of studies made by Hopkins (7) and by Davis and Pallesen (3) of the effects of rainfall on yield. The former in his study of Marquis wheat, and the latter in their study of spring wheat, found that additional rainfall occurring more than 90 days after seeding tends to reduce the yield of wheat. In his summer fallow and stubble series, Hopkins found no such detrimental effect although the benefits of late rains were much less than those resulting from additional rainfall earlier in the season. The author suggests that the detrimental effect illustrated in the Marquis study may result from lodging, a factor that would not influence protein content. It seems reasonable to assume that Hopkins, like the present authors, is unable to explain diminished yield with increased late rainfall in terms of a differential effect of increased rainfall on rates of translocation and respiration.

One other feature of the data deserves close attention. It will be observed that there is considerable similarity between the average rainfall curve of Fig. 1 and the regression curve of Fig. 2. Additional rainfall obviously has its greatest effect at periods when the average rainfall is low. This suggests that a given increment of rain occurring in April has a greater effect on protein content than an equal increment occurring in June, merely because rain is much scarcer in April. There can be little doubt that this hypothesis is essentially true, but the extent to which it serves to explain the difference between the effectiveness of April and June rainfall must await the results

of further investigation. In the meantime it should be borne in mind that the results of the present investigation stand independently of any explanation of them: under the conditions studied it appears that additional rainfall in April, early May, or late July, is more effective in reducing protein content than additional rainfall at other periods of the year.

An investigation of the sort reported in this paper presents many difficulties. The imperfections of the data are all too apparent and the propriety of analysing them by means of the selected statistical method may be open to question. Having accepted these difficulties, the application of the method leads inexorably to the production of a broad generalization, in this paper, the curve in Fig. 2. Like all generalizations it must be qualified, it applies only under certain conditions, it is not even fully representative of the original data since these contain various contradictions and exceptions not apparent in the final broad generalization. But the last difficulty is still the greatest. Having obtained the curve representing the effect of additional rainfall on protein content, it must still be interpreted. Whereas previous procedures are objective and mechanical, the last step is not. However, there is this to be said, that the responsibility for interpreting the curve can be shared with the reader.

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ARE THERE LIVING BACTERIA IN PERMANENTLY FROZEN SUBSOIL?¹

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Abstract

Samples of frozen subsoil were obtained aseptically at Churchill, Man., located on Hudson Bay at about 58° north latitude. These were cultured on various media. The procedure followed failed to demonstrate the presence of viable micro-organisms in the permanently frozen zone of a clay subsoil. Bacteria were isolated from samples obtained as deep as nine feet from the surface of a coarse gravel subsoil.

Introduction

Soil is a place of life—a dynamic mass harbouring large populations of many different forms of micro-organisms. These minute living creatures feed upon the mineral salts of the soil and the remains of plants and animals, and produce physical and chemical changes of great importance to agriculture. They are most numerous in fertile soil and in its surface layer, where organic matter abounds. Early investigators were not in agreement as to the presence of living bacteria deep in the subsoil. More recent work has shown that bacteria and fungi can be isolated from samples taken at considerable distances from the surface. Brown and Benton (1), after presenting a list of workers who have reported on the occurrence of bacteria at different depths of soil, recorded numbers ranging from 2100 to 81,600 bacteria and from 11 to 325 fungi per gram of air-dry soil in the C₂ horizon of 29 profiles sampled. These represented different soil types and dates of sampling from virgin and cultivated soils in Iowa. The depth of this horizon varied between 24 and 50 in. In a somewhat similar study with virgin soil in Manitoba, Timonin (13) in 1935, reported counts from the C horizon ranging from 20,500 to 6,000,000 bacteria and from 40 to 1840 fungi per gram of moisture-free soil in 10 profiles sampled. In this case subsoil was obtained between depths of 17 and 44 in. in the various profiles. Further, this investigator presented counts of 48,000 and 63,500 bacteria and of 500 and 875 fungi, respectively, per gram of moisture-free soil in two samples of the heavy blue clay obtained 64 and 76 in. below the surface of a peat soil. In neither of the investigations referred to was any sample taken at these depths found to be free of bacteria or of fungi. Furthermore, the decrease in count from the horizon immediately above in each profile was relatively small, which would strengthen the belief that considerable numbers of these organisms exist at much greater depths, and in fact, as deep as the soil itself.

¹ Manuscript received January 8, 1942.

Contribution from the Department of Bacteriology and Animal Pathology, The University of Manitoba, Winnipeg, Man. With financial assistance from a grant made by the late James A. Richardson, administered by the Research Committee of the university.

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Since there are vast areas where the subsoil is frozen and evidently has remained so for centuries, a study of these soils might be expected to reveal information of fundamental scientific interest in relation to (1) the possibility of micro-organisms withstanding long periods of dormancy and (2) the possible presence of types capable of carrying on normal metabolism in this frozen medium. Positive proof of dormancy would depend upon acceptance of the fact that the organisms were in the soil at the time it was frozen and upon suitable procedures for recovering them. It would be conditioned by satisfactory evidence that the chances of recent penetration by surface organisms and contamination due to faulty technique during sampling could be ruled out. A negative result, on the other hand, might mean merely that the portion sampled did not contain bacteria at the time of freezing. Or, it might mean that proper conditions for culturing the organisms in the soil were not provided in the procedure followed. A negative finding could be interpreted only in relation to the conditions of the experiment, and would have no bearing on that to be expected with another medium or set of incubation conditions. Obviously, a larger number of negative results, under different procedures, would have more weight than a single observation. Proof of a flora capable of reproducing in a frozen subsoil would involve the demonstration of reproduction *in situ*, which presents many difficulties. Or it would depend upon evidence that numbers of micro-organisms in one area changed significantly from time to time. This would necessitate working on samples immediately after obtaining them, or provision for transporting them under conditions that would prevent change in numbers during transit. Too, it would mean that the method used to estimate numbers of organisms in soil is capable of producing a result that can be accepted as correct.

The presence of micro-organisms in low temperature areas has been demonstrated. Darling (2) isolated many common species of spore-forming and non-spore-forming bacteria, as well as yeasts and moulds, from samples of soil and of snow collected during the second Byrd Antarctic expedition.

Issatschenko and Simakowa (4) reported on the bacteria of Arctic soils. They isolated several groups of bacteria common to temperate climates, and suggested that they might represent specific strains developed at low temperatures. Kazansky (6) studied the microflora of the soils of the Nova Zembla, obtained at 73° 6' north latitude. The author distinguishes seven types of soil in the area investigated. One of these, designated the flood-plain tundra, is found on a layer of permanent congelation and is not deeply embedded (20 to 40 cm.). It is almost black and of viscous consistence, with a good vegetative cover. Unfortunately, the data on numbers and groups of bacteria presented in his tables do not apply to this type of soil. This would have been of direct interest in the subject under consideration in this paper. He presents counts of bacteria by the microscopic method in profile studies on three of these types of soil, which confirm the belief expressed earlier that organisms exist as deep as the soil itself. Studies on various media reveal that the population consists of many kinds of bacteria, including

anaerobic and aerobic cellulose decomposers, nitrifiers and denitrifiers, nodule bacteria, and anaerobic nitrogen fixing bacteria. *Azotobacter* has not been detected. Levinskaya and Mamincheva (7) carried out an extensive study of the microbiological processes taking place in soils of Murmansk. Holes were dug to a depth of one metre in virgin soils, representing peat, sand, and sand-clay types. Samples from different depths were taken at one- to two-month intervals. Various media were used for the growth of different physiological groups of micro-organisms and for total counts. The results show that the population is small in these soils. It is highest in the sandy-clay soils (100,000 to 157,000 per gram of soil) and in samples obtained in the winter. The physiological processes are extremely slow and weak in intensity, showing a certain increase in the winter and autumn seasons. The authors attribute these evidences of low microbial activity to the low pH (5.2 to 6.4) of the soils investigated and to the influence of prolonged low temperatures. The population is smaller in the lower depths and the physiological activity weaker. The kinds of bacteria encountered are the same at all depths. *Azotobacter* was not detected in any of the studies. The same was true of members of the *Escherichia coli* group and the urea decomposing group. This prompted the authors to express the opinion that they had really selected virgin soils, that the micro-organisms isolated are inherent in soils of northern latitudes, and are cosmopolitans, adapting themselves to temperature, light, and soil conditions. Kapterev (5) reported on an investigation with frozen subsoil, carried on at 53° 58' north latitude in Siberia. He took samples aseptically from the sides of pits and from borings in frozen subsoil, transferred them to distilled water and incubated them for several weeks. The presence of living organisms in the samples was demonstrated repeatedly in his experiments. These represented several genera of unicellular and filamentous algae, moss, and a lower crustacean.

Lipman (8-12) has done much to create interest in the possibility of bacteria existing for extreme periods of time under conditions where accepted ideas about bacterial metabolism could not apply. Within the last decade this investigator reported having found viable bacteria in pre-Cambrian rocks, anthracite coal, stony meteorites, and in ancient rock structures erected by man; he suggested that these organisms must have existed in a quiescent state for long periods of time,—even for millions of years. Since his claims represent a direct contradiction of much that is taken for granted about living organisms, his work has been subjected to critical examination.

Permanently Frozen Subsoil at Churchill, Man.

The subsoil at Churchill, Man., which is located on the Hudson Bay at about 58° north latitude, is frozen. The country is a vast plain, treeless for miles in some areas and supporting dwarfed trees—poplars, spruce, tamarack—and swamp bushes in others. The surface is marshy, the thick layer of moss providing insulation which prevents thawing for more than a few feet at the surface during the short summer seasons. Underneath is to be found heavy

clay, which in some places is not more than a foot below the surface, while in other areas the bog seems to be bottomless. Official data on the temperature of the subsoil in this locality are not available. Residents are satisfied that at no time in the past 20 yr. has the thaw exceeded three feet. The accompanying table* presents data on air temperature at Churchill, which add

TABLE I
TEMPERATURES IN DEGREES CENTIGRADE, CHURCHILL, MAN.

Year	Jan.	Feb.	Mar.	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Mean												
1932	-24.4	-28.9	-24.4	-10.6	-0.6	6.1	12.2	16.1	7.2	-3.3	-13.3	-25.6
1933	-28.9	-28.9	-20.0	-12.2	-2.2	4.4	12.8	—	7.2	-3.9	-20.6	-31.1
1934	-26.7	-27.2	-22.2	-10.0	-3.9	4.4	12.8	10.0	4.4	-0.6	-12.2	-24.4
1935	-33.9	-20.6	-22.2	-10.0	2.2	4.4	11.7	11.7	5.6	-5.0	-18.9	-22.2
1936	-31.7	-30.6	-18.9	-15.6	-4.4	5.0	14.4	12.2	6.1	—	-16.7	-25.0
1937	-29.4	-23.9	-18.9	-6.1	3.3	7.2	14.4	11.7	6.1	-1.7	-14.4	-25.0
1938	-24.4	-25.0	-17.8	-13.9	1.1	8.9	12.8	11.1	8.9	0.6	-14.4	-17.8
1939	-27.8	-31.7	-25.0	-10.0	-1.1	4.4	11.7	10.6	4.4	-5.6	-11.7	-16.1
1940	-21.1	-23.3	-18.9	-10.0	0.0	6.1	13.9	12.8	10.0	-1.1	-16.7	-23.3
1941	-26.1	-22.2	-18.9	-9.4	-1.1	6.7						
Maximum and minimum												
1932	-5.0 -38.9	-10.0 -42.2	-11.1 -35.6	3.3 -27.8	24.4 -21.1	22.2 -2.8	30.0 -0.6	28.9 6.1	20.0 0.0	6.1 -11.1	3.3 -31.7	-5.0 -35.6
1933	-10.0 -37.2	-13.9 -41.1	5.0 -38.3	6.1 -27.8	12.2 -18.3	22.2 -3.3	27.8 1.1	— —	22.2 -1.1	7.8 -20.6	-3.3 -33.3	-17.2 -42.2
1934	-5.0 -37.8	-7.8 -38.3	-4.4 -35.0	3.9 -28.9	9.4 -22.8	20.6 -3.3	28.3 3.3	24.4 2.8	22.8 -5.6	11.1 -14.4	0.6 -27.8	-4.4 -43.9
1935	3.9 -45.0	-1.7 -35.0	-2.8 -36.7	11.1 -29.4	28.9 -13.3	22.2 -3.3	28.9 1.1	24.4 1.7	19.4 -3.9	10.6 -18.9	-3.3 -32.8	-1.7 -36.7
1936	-15.6 -43.9	-11.1 -43.9	-3.3 -33.9	2.8 -31.7	8.4 -22.8	23.9 -3.3	29.4 3.3	25.0 5.6	18.9 -2.8	5.0 -22.8	2.8 -30.6	-5.0 -42.2
1937	-16.7 -40.6	-0.6 -37.8	-1.7 -35.6	10.6 -25.0	20.0 -8.4	26.7 -1.1	27.2 3.9	25.6 0.0	22.2 -1.7	8.4 -12.2	-2.2 -27.8	-11.1 -38.3
1938	-8.4 -37.8	-2.8 -37.2	2.2 -31.7	6.1 -30.6	18.9 -10.6	30.0 -3.3	27.8 3.9	22.2 3.9	22.2 -0.6	16.7 -16.1	-0.6 -28.3	-1.7 -31.7
1939	-15.0 -38.9	-16.7 -40.0	0.0 -40.0	5.6 -24.4	17.8 -12.2	23.3 -4.4	26.1 2.2	25.0 0.0	18.3 -3.9	7.2 -17.8	2.8 -25.6	1.1 -31.7
1940	0.0 -37.8	-7.8 -35.6	-3.3 -37.2	5.0 -26.1	21.1 -16.7	18.3 -2.8	25.0 5.6	28.9 2.8	28.9 0.6	9.4 -14.4	0.6 -30.0	-9.4 -35.0
1941	-7.2 -41.1	-3.9 -36.1	-2.8 -36.7	7.2 -30.6	12.2 -12.2	26.7 -3.9						

* Courtesy of Winnipeg office, Air Services, Meteorological Division, Department of Transport, Canada.

weight to the opinion that the subsoil in this area has remained frozen for a long time. The flatness of the country, coupled with its swampy nature, makes the problem of obtaining samples of subsoil free from contamination difficult. Water seeps into any excavation made during the summer and carries with it types of micro-organic life characteristic of the surface layer. This difficulty could be obviated by working back from the face of a railroad cutting, where artificial drainage carries off the surface water. Only one such cutting could be found within 100 miles of Churchill. Since there is no other means of land communication to the port, the discovery of suitable sampling places is the first major difficulty to be overcome in any attempt to obtain valid information on the microscopic life in this district. Excavating heavy clay soil, frozen solid, in an area so far removed from industrial centres that the cost of machine digging or boring would be very high presents another serious problem. The hole must be large enough to permit working with aseptic technique while sampling, and deep enough to provide a fair margin below the thaw level. Recent penetration as a factor that might introduce microscopic life into frozen subsoil can be ignored. Surface water that seeps down to the frozen zone would freeze immediately and form a layer impervious to water. The subsoil beneath this could not be affected by the seepage.

Procedure

A clay cutting 8 ft. deep, located at Mile 505 on the Hudson Bay railway, was sampled on June 18, 1941. The clay was frozen immediately below the moss layer, which at this point was about 1 ft. thick. A pit 6 ft. deep and extending 8 ft. back from the base of the slope of the cutting was dug. Then a large chisel, sterilized over an open fire, was used to make horizontal excavations at three levels in the wall of the pit. Sterile pieces of steel, brought from the laboratory for the purpose, were used to dig about 10 gm. of soil from the top surface of an excavation and transfer it to a sterile test tube. Two samples were taken at each level, a different sampling tool being used for each. A fourth pair of samples was obtained from the clay immediately under the moss layer. As a check on the technique of sampling, soil sterilized in Petri plates in the laboratory was transferred to test tubes in the pit at approximately the same levels as the horizontal excavations. Further, plates containing sodium albuminate agar and others with Czapek's agar were exposed to the air of the pit for two minutes.

A second pit was dug on the slope of a gravel ridge rising about 50 ft. above the level of the surrounding country and extending for some miles parallel to the shore line of Hudson Bay. The ridge had been treed with spruce and was cleared only a few years ago, at the time of constructing the railway terminal and port. The sod and moss layer was about 2 ft. deep at the place excavated. Roots penetrated to a depth of 5 ft. The gravel was coarse and frozen in chunks, but not to a solid mass. It was readily loosened with an ordinary pick and a shovel. A thermometer inserted two inches into the wall at the 3 ft. level registered 2° C.; and at 6 ft. 0° C. This pit was

11 ft. deep and at the bottom extended back 12 ft. from the slope of the ridge. Samples were taken at four levels by the procedure described earlier.

The test tubes were packed carefully and transferred to the laboratory without refrigeration. Consequently, actual numbers of micro-organisms could have little significance because of the possibility of increase during the two weeks' exposure to summer temperatures. In order to minimize contamination that might take place in the laboratory during weighing, about half of each sample was transferred directly to a 50 ml. sterile dilution blank, the other portion being kept for further study. The initial dilution was shaken thoroughly, and a second dilution made by transferring 1 ml. to a 99 ml. blank. Transfers of 1 ml. were made in duplicate from each of the dilutions, representing approximately 0.1 and 0.001 gm. of soil, respectively, to the following media for culturing:

Difco nutrient agar in plates.

Sodium albuminate agar in plates; Med. 5 (3).

Amino acids-salts-glucose agar in plates (14).

Growth factors-salts-glucose agar in plates (14).

Nitrogen-free mannitol agar in plates; Med. 77 (3).

Sodium-nitrate-sucrose agar in plates; Med. 16 (3).

Peptone-glucose acid agar in plates; Med. 18 (3).

Difco nutrient broth in tubes, for protozoa.

Hay infusion in tubes, for protozoa.

Ammonium sulphate-cellulose solution in tubes; Med. 85 (3).

Glucose-peptone agar in deep tubes for anaerobes; Med. 12 (3).

Calcium nitrate solution (Detmer's) in tubes for algae; Med. 42 (3).

Thiosulphate* solution for sulphur oxidizing bacteria; Med. 65 (3).

Iron-ammonium* sulphate solution for iron oxidizing bacteria; Med. 74 (3).

Incubation was at 25° C. for one week for all agar plate studies and for protozoa, and for about one month for cellulose decomposers, anaerobes, sulphur oxidizing and iron oxidizing bacteria. The cultures for algae were incubated in a sunny window at summer temperature for six weeks. This procedure provided 24 results on the frozen subsoil and eight on the surface soil for each medium used with each type of soil studied. These represented duplicates from two dilutions from each of two samples taken at four different levels. The sterile soil transferred in the pit was studied in the same way, on the 0.1 dilution only.

Results from Excavation 1—Clay Cutting

Except for a few colonies, which clearly represented air contaminants since they did not appear on all plates in a set and were equally numerous from the 0.1 and 0.001 dilutions, all results for samples taken at depths of 54 and 40 in. were negative as were those also for 24-in. samples, except in the medium for anaerobic bacteria. All of the four transfers from 0.1 dilutions and one

* After transfers to the above media had been made about half of the remainder of the initial dilution was added to 50 ml. of each of these two media in 150 ml. Erlenmeyer flasks.

from a 0.001 dilution produced colonies and gas in this medium. Samples taken at the 12 in. level, just below the moss where the soil was not frozen at the time of sampling, gave positive results in all media, except those for protozoa and iron oxidizing bacteria; counts ranged up to many hundreds of colonies per plate from the 0.001 dilutions on all agar media for bacteria, and up to 100 per plate from the 0.1 dilutions on media for fungi. Three transfers from 0.1 dilutions, but none from 0.001 dilutions, showed evidence of growth in the medium for anaerobic bacteria. The same number of transfers from 0.1 dilutions produced growth in the medium for algae. The activity of cellulose decomposers was evident in all tubes of media containing 0.1 dilutions, and in two of the four tubes containing 0.001 dilutions. The sulphur oxidizing medium supported motile short rods from all dilutions, the growth appearing white and flaky. All of the media containing transfers from the 0.1 dilutions of sterile soil, used to check the technique of sampling at the various levels in the pit, gave negative results. On the other hand, plates exposed two minutes to the air of the pit contained many colonies, in general appearance not unlike types of fungi and of bacterial colonies usually encountered as air contaminants.

Results from Excavation 2—Gravel Ridge

The results for this excavation were quite different from those for the clay cutting. Samples taken at a depth of 9 ft. produced many colonies, uncountable for the 0.1 dilutions and varying from 76 to 375 for the 0.001 dilutions on all plates prepared with the various agar media for bacteria. Two of the transfers from 0.1 dilutions gave positive results in the medium for anaerobic bacteria. In the media for cellulose decomposing bacteria, sulphur oxidizing bacteria, iron oxidizing bacteria, fungi, algae, and protozoa, there was no evidence of growth from any of the dilutions. The results for samples taken at 6 ft., 4 ft., and 1 ft. in general were in agreement with those at the 9 ft. level. Bacteria that developed on the plating media were more numerous in the higher level samples. A few fungi and cellulose decomposing bacteria were found in the samples taken at a depth of 4 ft. The 1-ft. level samples contained about the same numbers of organisms as the surface samples taken from the excavation in clay soil.

Discussion

The apparent discrepancy in the findings from the two excavations may be attributed to the difference in permeability of the two types of subsoil. Undoubtedly, the loose textured coarse gravel would permit thawing and seepage of surface water to a greater depth than would clay subsoil. The presence of bacteria in samples of the gravel subsoil cannot be accepted as an indication of their ability to withstand long periods of dormancy in a frozen medium. The organisms may have been carried there relatively recently in seepage water. The failure to demonstrate the presence of fungi in these samples weakens this statement, since one would expect fungal spores to be

found in contamination from surface water. The data published by Brown and Benton (1) and Timonin (13) indicate that the proportion of fungi to bacteria in the C horizon of unfrozen subsoil may not differ widely from that in the surface layer of the same profile.

It is obvious that a statement on either of the theories that prompted this study is not justified by the results obtained. This report merely presents some of the problems encountered and the findings obtained in a preliminary investigation.

Acknowledgments

The authors are pleased to express their appreciation to Dr. A. H. R. Buller, Professor Emeritus of Botany, The University of Manitoba, for suggesting this problem, obtaining financial support for the expedition and for his inspiring interest in the project; and to Dr. M. I. Timonin, Agricultural Scientist, Division of Bacteriology and Dairy Research, Department of Agriculture, Ottawa, Canada, for his assistance in locating and translating the references in Russian.

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STUDIES IN TREE PHYSIOLOGY

III. THE EFFECT OF THE METHOD OF CUTTING ON THE WATER CONTENT OF TWIGGS. A NOTE ON A PAPER BY McDERMOTT¹

BY R. DARNLEY GIBBS²

Abstract

Significantly higher water contents are found in short samples of twigs isolated by simultaneous cuts than in samples isolated by consecutive cuts in which displacement of water obviously takes place. This is in agreement with the results of McDermott. Natural water gradients are small and do not invalidate McDermott's figures. Displacement is not confined to the immediate neighbourhood of the cut.

Introduction

In a previous paper (2) the author concluded that most of the available evidence is in favour of the tension hypothesis of the ascent of sap in trees. If it is true that columns of water are drawn up from above then they must be under tension. That this is indeed the case seems to be borne out by observations on the rapid movements of dyestuffs introduced into newly-cut vessels and perhaps by the diametral changes associated with fluctuations in transpiration, though an alternative view—that vessels may at times contain gas at less than atmospheric pressure, rather than water under tension—has been put forward by Priestley (4).

It is clear that whether vessels contain water alone under tension or water and gas under reduced pressure, there will be a movement of water when the vessels are cut. The amount of movement, however, might differ greatly in the two cases. In the former an extremely small change might relieve the tension (since water is practically inextensible) unless the vessels are greatly contracted by the tension. Their expansion would then withdraw a considerable amount of water from the cut ends. If many of the vessels contain gas under reduced pressure cutting would be followed by the entrance of air but not by any great movement of water from near the cut end, unless individual vessels have both gas *and* water in them.

In early determinations of water contents (1) the danger of considerable change during cutting was regarded as a distinct possibility and some attempt to eliminate it was made by removing small cores of outer sapwood with a hollow punch. This, driven into the tree with a single heavy blow, isolated a short cylinder of wood without possibility of vertical displacement. Results from this led to the belief that changes were too small to affect seriously the determinations, but no extensive study of this was made.

A recent paper by McDermott (3) has reopened the question. McDermott isolated small sections from twigs by two simultaneous cuts, thus preventing

¹ *Manuscript received November 17, 1941.*

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withdrawal of water. He compared the water contents of such pieces with those of adjacent parts of the twigs taken by consecutive cuts both above and below the initial cuts. Significantly lower water contents were obtained for the latter. He concluded that these lower values were due to the displacement of water resulting from the release of tensions.

Although his results seem convincing he appears to have neglected one perhaps remote possibility—namely, that the natural gradient of water content in a twig might be such that (especially if single internodes were used) the middle piece would contain more water than the adjoining distal and proximal pieces.

It is not absolutely clear from McDermott's work that he used more than one internode in every sample, but his three samples were each about 3 in. long which would seem to make this certain.

Materials and Methods

In the present work, twigs of *Tilia*, *Ulmus*, and *Fraxinus* were used. In *Tilia*, samples were taken from single internodes of twigs of the current year's growth (Fig. 1b). The twigs of *Ulmus* and *Fraxinus* were older and individual samples extended over more than one internode, although they were about the same length as those of *Tilia* (about 1 in.).

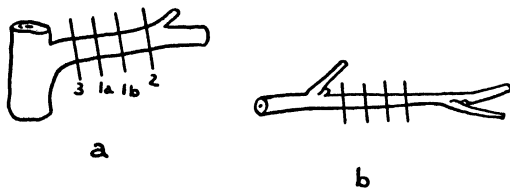


FIG. 1. Diagrams showing methods of taking samples. (a) McDermott's method: middle piece isolated by simultaneous cuts, proximal and distal pieces removed by consecutive cuts. (b) Present method with *Tilia*: proximal, middle, and distal pieces are from a single internode.

As in McDermott's work three samples were taken from each twig, but in order to eliminate any possibility of error due to existing gradients, the cuttings were made in such a way that sometimes the distal, sometimes the middle, and sometimes the proximal samples were made with simultaneous cuts, the others being made with consecutive cuts. No more than a few seconds elapsed between the initial cuts and the final bottling of all three samples in weighed rubber-stoppered tubes.

The first two experiments (B.G. 41.3 and 41.5) were carried out in the early afternoon, using twigs from young trees of *Fraxinus americana* L. and *Ulmus americana* L., growing in the Montreal Botanical Garden. The weather was hot and sunny and tensions presumably high, though no check of this was made. The results are summarized in Tables I and III.

McDermott carried out some experiments in which samples were taken on a dull, moist day, when transpiration presumably was slow and tensions

TABLE I

WATER CONTENT (%) OF TWIGS OF *Ulmus americana* AND *Fraxinus americana*; SAMPLES TAKEN FROM DISTAL, MIDDLE, AND PROXIMAL POSITIONS*

Twig	<i>Ulmus americana</i> : 1 to 2 p.m., July 14, 1941. (Expt. B.G. 41.3)			<i>Fraxinus americana</i> : 2 to 3 p.m., July 24, 1941. (Expt. B.G. 41.5)		
	Distal	Middle	Proximal	Distal	Middle	Proximal
1	65	63	64	65	63	62
2	71	73	69	58	61	56
3	77	76	78	66	65	64
4	74	70	70	65	59	58
5	73	76	71	59	60	56
6	78	76	79	65	63	65
7	94	86	87	67	62	60
8	72	73	72	69	70	67
9	71	69	70	62	63	59
10	76	71	74	62	67	61
11	70	75	65	61	63	60
12	81	80	85	68	67	70
Means	75.2	74.0	73.7	63.9	63.6	61.5
Means: samples taken with simultaneous cuts			76.5	64.25		
Means: samples taken with consecutive cuts			73.2	62.4		

* Figures in bold-face type are from samples made with simultaneous cuts; the others from samples obtained with consecutive cuts. Each twig came from a separate tree. All results are calculated on a dry-weight basis.

small. He obtained significantly smaller differences in water content than under conditions of rapid transpiration. In view of McDermott's results, further experiments, D.H. 41.5 and 41.6, were carried out. Since at night transpiration is reduced, and tensions probably are lower, one set of observations was made in the early afternoon of a warm, clear day and another set at dawn the next morning. Single internodes from current growth on young trees of *Tilia americana* L. were used. The results are summarized in Tables II and III.

Results and Discussion

The results of experiments B.G. 41.3 and 41.5 are summarized in Tables I and III. Two facts are indicated by the figures given.

1. There appears to be a slight gradient in the water content of twigs of both trees, the distal samples containing more and the proximal samples less water than the middle pieces. While these gradients are not both statistically significant when considered separately, they are significant when considered as a group with the similar results from *Tilia* (P is <0.001).

2. Significantly higher values are obtained when samples are taken with simultaneous cuts than when they are made with consecutive cuts. This is in agreement with McDermott's findings.

The results of experiments D.H. 41.5 and 41.6 are summarized in Tables II and III.

TABLE II

COMPARISON OF WATER CONTENT (%) OF TWIGS OF *Tilia americana* IN EARLY AFTERNOON AND AT DAWN; SAMPLES TAKEN FROM DISTAL, MIDDLE, AND PROXIMAL POSITIONS*

Twig	<i>Tilia americana</i>					
	1 to 2 p.m., Sept. 15, 1941. (Expt. D.H. 41.5)			5 to 6 a.m., Sept. 16, 1941. (Expt. D.H. 41.6)		
	Distal	Middle	Proximal	Distal	Middle	Proximal
1	123	113	109	136	117	113
2	112	129	107	123	125	111
3	112	110	120	122	117	123
4	132	115	111	136	115	108
5	106	121	103	105	107	94
6	115	112	120	129	127	131
7	99	86	86	122	104	102
8	117	130	117	124	129	113
9	114	112	120	137	130	132
Means	114.4	114.2	110.3	126.0	119.0	114.1
Means: samples taken with simultaneous cuts			121.6	126.8		
Means: samples taken with consecutive cuts			108.7	116.2		

* Figures in bold-face type are from samples made with simultaneous cuts; the others from samples obtained with consecutive cuts. Each twig came from a separate tree. All results are calculated on a dry-weight basis.

TABLE III

ANALYSIS OF VARIANCE

Tree	Source of variance	D.f.	Sum of squares	Variance	F	P
<i>Ulmus</i>	Between twigs	11	1385.89	125.99	49.41	$\ll 0.01$
	Between positions	2	14.89	7.44	2.92	> 0.05
	Simultaneous vs. consecutive cuts	1	88.89	88.89	34.86	$\ll 0.01$
	Residual (error)	21	53.55	2.55		
<i>Fraxinus</i>	Between twigs	11	363.33	33.03	11.67	$\ll 0.01$
	Between positions	2	41.17	20.58	7.27	< 0.01
	Simultaneous vs. consecutive cuts	1	28.12	28.12	9.94	< 0.01
	Residual (error)	21	59.37	2.83		
<i>Tilia</i> (afternoon)	Between twigs	8	1976.67	247.08	53.71	$\ll 0.01$
	Between positions	2	96.22	48.11	10.46	< 0.01
	Simultaneous vs. consecutive cuts	1	988.17	988.17	214.82	$\ll 0.01$
	Residual (error)	15	68.94	4.60		
<i>Tilia</i> (dawn)	Between twigs	8	2086.96	260.87	74.75	$\ll 0.01$
	Between positions	2	642.74	321.37	92.08	$\ll 0.01$
	Simultaneous vs. consecutive cuts	1	675.57	675.57	193.57	$\ll 0.01$
	Residual (error)	15	52.36	3.49		

As in *Fraxinus* and *Ulmus*, a gradient appears to exist, with slightly higher water contents at the distal end. Significantly higher figures for water contents were also obtained from samples made with simultaneous cuts than from those made by consecutive cuts. This was true of the twigs cut at dawn as well as of those cut in early afternoon, though the difference was (on the average) slightly smaller at dawn.

A question that arises from work of this kind is—how far from a cut does displacement have measurable effect? An analysis of the figures given in the tables shows that, where the sample made with simultaneous cuts was the end one (either distal or proximal), of the three pieces taken it had a higher water content than the other two pieces; the latter, however, had almost exactly the same water contents (average for all species: 90.95% for the pieces next to the first piece and 91.07% for the remote pieces). It is true, of course, that the first figure is obtained from distal and proximal samples only, while the second is from distal, proximal, *and* middle pieces. That this does not affect the result is evident from the fact that the average water content of *all* distal and basal pieces taken together is almost exactly the same as that for *all* middle pieces (92.4 and 92.7%, respectively).

Acknowledgments

The trees used in the present investigation came originally from the Provincial Forest Tree Nursery at Berthierville, Que., and were the gift of the Provincial Government through the kindness of the Chief of the Forest Service, Mr. G. C. Piché. Some of them have been growing at the Montreal Botanical Garden during the past three years by courtesy of the Director, Professor Marie-Victorin. Mr. George Pickrell gave assistance in cutting the *Ulmus* and *Fraxinus*. Dr. A. Steinberg's generous help made possible the statistical tests. The author thanks these gentlemen for their co-operation.

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STUDIES ON ROOT ROT OF CORN IN ONTARIO¹

By J. K. RICHARDSON²

Abstract

Root rot of corn in Ontario is caused primarily by parasitic soil micro-organisms, the most important of which are species of *Pythium*, *Helminthosporium*, and *Fusarium* in that order. The disease causes a decrease in the stand by pre-emergence killing and a dwarfing of the plants by the parasitic invasion and destruction of their roots by the organisms. The pathogens have different optimum soil temperatures but the lower ranges favour those that cause the most severe damage. The roots of other field crops can be parasitized by the organisms found associated with corn root rot, but their effect on the development of the crop varies greatly. It has been proved under greenhouse conditions that the severity of the disease is greatly reduced if the corn is preceded by cover crops of soybeans and materially increased when preceded by timothy. Other crops tested have an intermediate effect.

Introduction

In some seasons, root rot may be a limiting factor in the growth of corn. In affected fields the stand is usually thin and the growth very uneven. By midsummer most of the plants are dwarfed and chlorotic. The examination of diseased plants reveals root systems rotted to such an extent that only a few secondary roots are able to function.

The present paper deals with laboratory and greenhouse studies of parasitic root rots of corn caused by species of *Pythium*, *Helminthosporium*, and *Fusarium*.

Literature Review

The earlier conceptions of corn root rot were that the organisms that caused stalk and ear rots were also able to infect and rot the roots when introduced into the ground by means of diseased seed. This theory was advanced in the Central States by Holbert and Hoffer (10) in 1920 and also by Koehler and his associates (15) in 1924, the latter stressing the fact that since corn and wheat are both subject to the ravages of *Gibberella Saubinetii* (Durieu & Mont.) Sacc., these crops should not follow each other in a rotation.

In 1923-24, Hoffer and Carr (9) working in Indiana, and Manns and Phillips (16) in Delaware, were convinced that the root rot condition in corn was associated with soil deficiencies or poor drainage and lack of fertility.

In Connecticut, in 1919, Clinton (3) observed oospores of a fungus, which he thought resembled *Phytophthora Cactorum* (Lebert & Cohn) Schr., in the bases of corn stalks affected with root rot, but did not isolate the fungus. Johann and her co-workers in Illinois and Wisconsin in 1926 (11, 12)

¹ Manuscript received January 2, 1942.

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attributed the disease to infection by a species of *Pythium*, later described by Drechsler (5) as *Pythium arrhenomanes* n. sp. Within the next several years Valleau *et al.* (21) and Branstetter (1) described similar root rots of corn and stressed their similarity to a root rot of sugar-cane in Hawaii, which was described by Carpenter (2) in 1921 and attributed by him to soil inhabiting *Pythium*-like fungi.

Melhus *et al.* (17) and Wen-Chun Ho and Melhus (8) in their recent researches on maize root rot in Iowa described the symptoms following infections by *Pythium graminicola* Subr. and *P. de Baryanum* Hesse and classified a large number of isolates in order of their importance as root parasites; among them *P. graminicola* and *Rhizoctonia Solani* Kühn were considered to cause severe injury, while *P. de Baryanum* and *Helminthosporium sativum* P. K. & B. were of lesser importance. Recently Wen-Chun Ho (7) suggested that there appears to be a succession of invasions by different species of fungi as the season advances. He also found a variation in the susceptibility of varieties of maize to some of the pathogenic organisms.

Materials and Methods

Corn seed after being selected for uniformity and freedom from disease was disinfected in a 1 : 1000 mercuric chloride solution and planted in steam sterilized and non-sterilized soil of three types (1) clay loam that had produced a badly rotted crop of corn, (2) light sandy loam on which brown root rot of tobacco had been severe, and (3) greenhouse compost. All unsterilized soils produced crops with typical root rot symptoms, while the corn growing in steamed soil was entirely free from the disease (Fig. 6).

Isolations were made from incipient infections on small roots of corn both from the field and from root rot soil in the greenhouse. These isolates were then tested on corn growing in artificially inoculated soil, to determine their parasitic capabilities.

Throughout the experiments inoculations were made by planting seed in sterilized soil in which was incorporated an inoculum, in volume equal to 5 % of the soil, of a pure culture of the isolates growing on a mixture of crushed oats and corn-meal.

Root sections for microscopic examination were taken from young incipient infections, cleared by heating in a solution of lactophenol (to which a small quantity of basic fuchsin had been added) and mounted in clear lactophenol.

Isolations and Preliminary Observations

By selecting incipient root infections for microscopic examination it was possible, in most instances, to identify the parasitic forms and obtain a fairly accurate conception of the flora present. In the diseased roots of corn were found a number of distinct types of organisms, many of which are commonly present in diseased roots of other species of plants. *Pythium* spp. in the form of lobulate sporangia and oospores and fine septate mycelium were most

commonly observed. The so-called phycomycetous mycorrhizal fungus (6, 13, 23) which formed a profusion of mycelial growth on the root surfaces, as well as arbuscule and vesicle development within the host tissues, was also in evidence. *Olpidium Brassicae* (Wor.) Dangeard (6, 19, 22) occurred frequently, often in rootlets free from visible discoloration. Both parasitic and saprophytic species of nematodes, mycelium of *Helminthosporium* and *Rhizoctonia* spp., mycelium and chlamydospores of *Fusarium* spp., fungi that could not be classified, as well as numerous areas of necrotic tissue apparently devoid of organisms, were also present.

In a preliminary series of inoculations on corn in artificially inoculated soil, involving all the organisms isolated from corn roots, only six proved to be definitely parasitic. These were a *Pythium* of the *Nematosporangium* group, probably *P. arrhenomanes* Drechs., *P. de Baryanum* Hesse, *Helminthosporium bicolor* Mitra, *H. Maydis* Nisikado & Miyake, and two species of *Fusarium*. These parasitic forms, which caused various degrees of injury up to severe necrosis and disintegration of root tissue, were used in subsequent experiments. Careful examinations of corn roots infected with pure cultures of the above organisms made it possible to determine the specific symptoms produced by each pathogen (Figs. 7 to 11) and the appearance of the organisms within the host tissues. These observations provided a basis for future examinations of the roots of corn and other host plants growing in naturally infected soil where the specific pathogenic factor was not known.

The Effect of Pathogens Isolated from Corn Roots on the Germination and Growth of Corn

Flats of sterilized soil were artificially inoculated with pure cultures of the above-mentioned organisms, seeded to corn, and kept at a temperature of 65° to 75° F. *Pythium arrhenomanes* and both *Helminthosporium* species affected the germination, causing a reduction of from 40 to 50% and 5 to 10%, respectively. In plants that developed, the leaf growth varied in direct relation to the degree of root damage induced by the pathogen in question. After 24 days, plants in sterilized compost and those in the flats inoculated with *Fusarium* spp. were from 12 to 14 in. high, those to which *P. de Baryanum* and sterile culture medium had been added were 8 to 11 in., while with *P. arrhenomanes* and *Helminthosporium* spp. growth was only 5 to 8 in. in height. In addition to the reduction in growth many plants in the flats inoculated with *P. arrhenomanes* were chlorotic and malformed, while a number of those inoculated with *Helminthosporium* spp. were infected above the ground on the coleoptile or first leaf.

In experiments in which sterilized culture medium was added as a control, there was invariably a retardation in growth, equivalent to, or even slightly more than where weak or moderate parasites had been used. This cannot be attributed to chance infection since the roots of such plants, though slightly less vigorous, were as free from discoloration as those growing in steamed soil. On the other hand, where non-parasitic isolates were used as inoculum,

both leaf and root growth were comparable to plants from the steamed soil series (Figs. 4 and 5).

The Effect of Parasitic Fungi Isolated from Corn Roots on Other Host Plants

Since there was a marked similarity in organisms found in the roots of corn growing in different soils, and it has been shown (6) that the same species of fungi may attack the roots of a variety of hosts, 11 field crops were tested for their susceptibility to infection by the pathogens isolated from corn to determine those best suited for rotation with that crop.

Wheat, oats, rye, timothy, soybeans, red clover, alfalfa, rape, buckwheat, sugar beets, tomatoes, and corn were grown in soil artificially inoculated with pure cultures of the fungi, and in addition to macroscopic observations on plant growth and root condition, careful microscopic examinations were made to determine the variation in parasitism on the respective host roots.

All plants were kept under similar environmental conditions in the greenhouse and although the artificially inoculated soil might tend to exaggerate the infection, particularly of the weaker pathogens, nevertheless the results

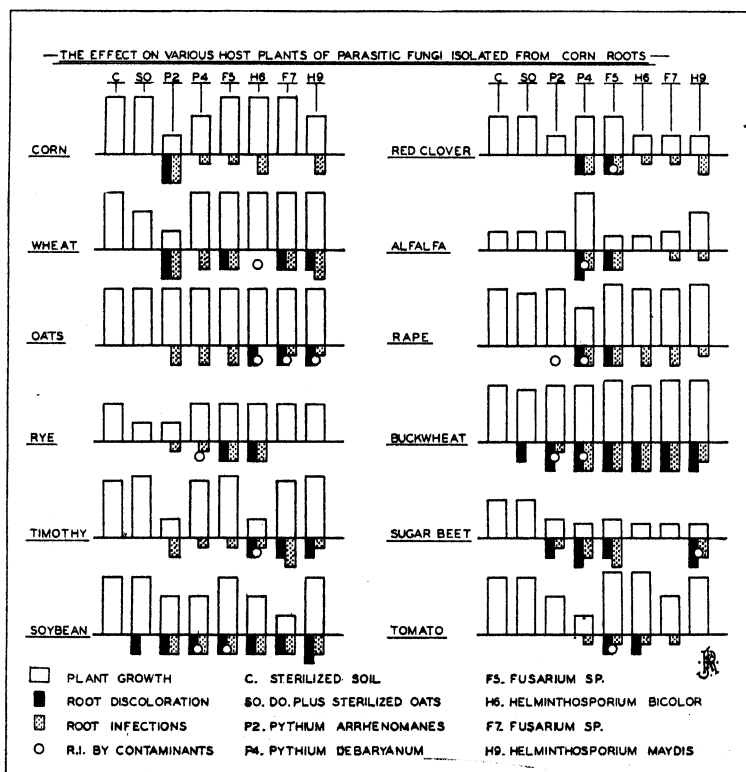


FIG. 1. *The effect on various host plants of parasitic fungi isolated from corn roots.*

obtained may be regarded as characteristic of the parasitic capabilities of the isolates tested. In Fig. 1 the condition of plants growing in sterilized soil (C) was taken as normal for the environment under which the experiment was conducted.

In corn, wheat, timothy, and sugar beet affected by P_2 and other plants in which affected root systems and significant reduction in growth coincided, there can be little doubt that the organisms concerned were responsible for the stunting. On the other hand in soybeans and buckwheat, for example, where there were severely discoloured and infected roots without material reduction in growth, the effect of the organisms may be considered as negligible.

Micro-organisms in the Roots of Various Crop Plants When Grown on Corn Root Rot Soil

Following the determination of the reactions of the pathogens isolated from corn roots on different host plants growing in artificially inoculated sterilized soil, a test was conducted to study the behaviour of these same hosts when growing on soil that had produced severe root rot of corn in the field similar to that illustrated in Fig. 3. No attempt was made to grow the plants to maturity, but periodic examinations were made of typical root lesions from each host to record the types of micro-organisms present.

Table I gives the general macroscopic appearance of the various plants as well as a record of the microscopic condition and fungus flora of their roots. It is reasonable to assume that similar conditions would prevail when these crops are grown on infected soil under natural field conditions.

Pythium spp., as shown by the presence of lobulate sporangia and oospores (1 and 2) and some of the mycelial invasion recorded (7), were the organisms most prevalent in the roots. Where this invasion is accompanied by root discoloration and abnormal growth, as in corn, wheat, timothy, alfalfa, and buckwheat, there can be little doubt that these crops when grown either immediately before or following corn, would tend to aggravate the root rot situation. It is interesting to observe that although no *Rhizoctonia* spp. were isolated from corn roots, these fungi must have been present in large numbers in corn root rot soil since in the present experiment when damping-off was evident, *Rhizoctonia* mycelium was observed in the roots of many of the plants. The other micro-organisms, although sometimes present in considerable quantities, do not appear to have any appreciable effect on the crop.

Cover Crop Experiments

Following the significant variation in the reactions of different crop plants when grown on root rot soil, two similar series of experiments were conducted in which four successive crops of each of 12 host plants in addition to five different four-crop rotations of groups of these same plants were grown (1) in corn root rot soil and (2) in steamed compost inoculated with a mixture of pure cultures of corn root pathogens. The leafy succulent growth from

TABLE I

THE DEVELOPMENT AND ROOT FLORA OF VARIOUS PLANTS WHEN
GROWING IN CORN ROOT ROT SOIL

Host	Condition of plants	Condition of roots													
		Macroscopic			Microscopic										
		A	B	C	1	2	3	4	5	6	7	8	9	10	
Corn	Stunting of growth	-	+	-	+	+	+	-	+	+	+	+	-	-	
Wheat	Chlorosis of leaf tips	-	-	+	+	+	-	-	+	-	-	-	+	-	
Oats	Normal growth	+	-	+	+	-	-	+	-	-	-	-	-	-	
Rye	Normal growth	-	+	-	+	-	-	-	-	+	-	-	-	+	
Timothy	Considerable damping-off	-	+	-	+	+	+	+	-	-	-	-	-	+	
Soybeans	Normal growth	-	+	-	-	-	+	-	-	-	+	-	-	+	
Red clover	Some damping-off	+	-	-	+	+	-	-	-	+	+	+	-	+	
Alfalfa	Severe damping-off	-	+	-	+	+	-	+	-	-	+	+	-	-	
Rape	Some damping-off	+	-	-	-	-	-	-	-	-	+	+	-	-	
Buckwheat	Fair growth	-	-	+	+	+	+	-	-	-	-	-	-	-	
Sugar beets	Severe damping-off	+	-	-	-	-	-	+	-	-	-	-	-	-	
Tomatoes	Slight damping-off	+	-	-	-	-	-	+	-	-	+	+	+	-	

NOTE.— A = apparently healthy roots, no discoloration.

B = slight discoloration or a few lesions.

C = severe browning or many lesions.

1 = *Pythium*, lobulate sporangia.

2 = oospores.

3 = *Helminthosporium mycelium* and spores.

4 = *Rhizoctonia mycelium*.

5 = *Olpidium brassicae*.

6 = mycorrhizal fungus.

7 = miscellaneous fungus invasion (not identified).

8 = nematodes and eggs.

9 = isolated necrotic areas.

10 = cell breakdown without visible fungus.

each crop was thoroughly incorporated with the soil in which it had grown and initial decomposition was allowed to occur before the succeeding crop was sown. Sixteen days after the incorporation of the last cover crop, corn was planted in each of the pots.

A complete record of the condition of the corn following each of the cover crops and rotations was made for each of the quadruplicate series of pots with both types of soils. In general there was a marked similarity in the behaviour of the corn on both the naturally and artificially infected soils. The growth of all the host plants was more vigorous on the steamed compost and there was a slightly greater variation in the diseased condition of the roots. Since the differences between the two series were entirely quantitative, only the averages of the groups growing in naturally infected soil will be considered.

Though it was impossible to obtain accurate numerical counts of the diseased roots, it was possible to obtain a reasonably accurate idea of the condition of the corn roots as a whole, as affected by the various preceding

crops. Numerical ratings were assigned to different degrees of infection according to the following schedule.

1. Only a few visible lesions on root laterals.
2. Slight infections on a number of laterals.
3. General infection on most laterals.
4. Most laterals infected and some destroyed.
5. Severe infection of laterals and lesions on the primary radicle.

These data and the corresponding height of plants are recorded in Table II. See also Figs. 12 to 14.

TABLE II

THE EFFECT OF VARIOUS COVER CROPS ON THE SUBSEQUENT
DEVELOPMENT OF CORN IN ROOT ROT SOIL

Successive crops			Rotations		
Preceding crops	Height, in., 18 days	Root infection rating	Preceding crops	Height, in., 18 days	Root infection rating
Soybeans	11.0	0	Rape	13.0	1
Red clover	11.0	3	Rye		
Oats	9.5	1	Timothy		
Wheat	9.5	2	Soybeans		
Alfalfa	9.0	2	Wheat	10.5	2
Rye	9.0	3	Alfalfa		
Tomatoes	8.5	1-	Sugar beets		
Corn	8.0	5	Oats		
Buckwheat	8.0	2	Soybeans	10.5	3+
Rape	7.5	1	Rape		
Sugar beets	7.5	2	Alfalfa		
Timothy	7.0	4	Buckwheat		
			Red clover	8.5	3
			Timothy		
			Wheat		
			Red clover		
			Buckwheat	7.5	5+
			Oats		
			Timothy		

Specimens of typically affected corn rootlets from each series listed in Table II were also examined to determine possible variations in their microflora. Although a comparatively small number of roots were examined following each series of cover crops, the type and quantity of material examined was comparable in each.

In the graph, Fig. 2, the size of the blocks for each particular type of invasion is proportionate to the total amount of infection in the roots examined. In the case of nematodes, the actual number present was recorded. Only the organisms within the host tissues were taken into consideration and

no attempt was made to differentiate between primary and secondary invaders.

In the root examinations no differentiation was made between the species of *Pythium* observed. Both *P. arrhenomanes* and *P. de Baryanum* were present, but the former was responsible for the greater number of infections. The oospores were also predominately *Pythium* spp., though it is conceivable that *Phytophthora* spp. may also have been present. It was difficult to positively identify much of the fine mycelium present, but a comparison with the infections produced in the pure culture inoculations previously recorded leaves little doubt that the majority of these could be attributed to invasions by *Fusarium* spp. No classification was made of the nematode populations, both parasitic and saprophytic forms being present.

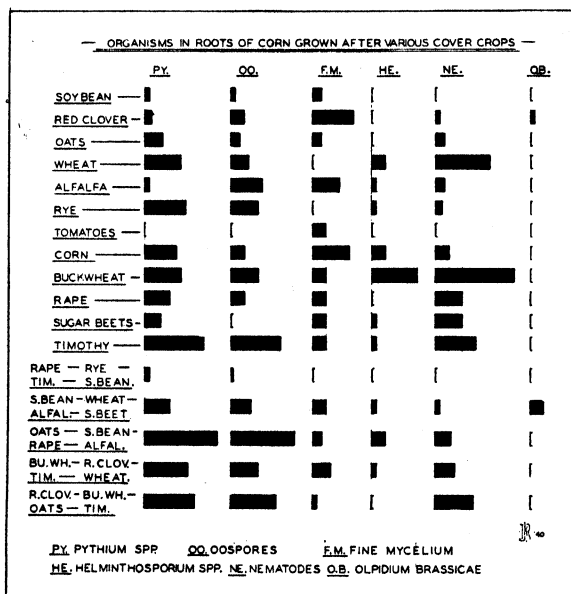


FIG. 2. Organisms observed in the roots of corn grown in root rot soil following various cover crops.

In a comparison of the root infection rating in Table II with the organisms observed in the roots as recorded in Fig. 2, it will be noted that in most roots the macroscopic condition is reflected in the amount of infection present as recorded from microscopic examination. In general, however, the records of microscopic examinations convey a somewhat erroneous impression, since the amount of infection present appears greater than the macroscopic root infection rating would indicate. In making microscopic examinations it was not possible to take into account the severity of infection for while similarly infected roots were examined it was sometimes necessary to examine the worst and sometimes the best of the diseased roots. For instance, when

corn followed soybeans, it was extremely difficult to find infected rootlets but in corn following timothy or corn, the difficulty was to find tissue that was not too severely infected for comparable examination. It is fully realized



FIG. 3. Field of corn severely affected by root rot.

FIG. 4. Corn after 24 days' growth in steamed compost inoculated with (1) *Pythium arrhenomanes*, (2) *P. de Baryanum*, (3) sterile culture medium (oats plus corn-meal), (4) not inoculated.

FIG. 5. Same as Fig. 4, inoculated with (1) *Helminthosporium Maydis*, (2) *H. bicolor*, (3) a non-parasitic isolant, (4) not inoculated.

that with the majority of specimens a very small percentage of affected rootlets was examined and additional examinations might have increased the preponderance of certain types of infections. However, since similar tissue was examined in all instances, it is reasonable to assume that a fairly accurate idea of the organisms responsible for the root damage was obtained.

This experiment shows beyond any doubt that the general vigour and condition of a crop of corn growing on root rot soil is influenced materially by the crops that precede it as indicated by the growth following successive plantings of the same crop. The most significant effect, however, appears to be produced from the crop immediately preceding, as demonstrated in the crop rotation series.

There is undoubtedly a certain stimulation derived from the growth of leguminous crops, such as soybeans, red clover, and alfalfa, but this advantage is not necessarily reflected in the condition of the roots. Following soybeans the roots of corn appear perfectly healthy and it was difficult to find even a trace of decay, whereas after red clover or alfalfa the roots were little cleaner than those grown in soil in which corn had been grown continuously. In contrast, timothy had the most severe detrimental effect, the reduction in growth and diseased condition of the roots being more severe than when corn had been grown continuously. All other crops tested had an effect intermediate between these two extremes. It is interesting to note that whatever the beneficial effect of soybeans on the following corn crop may be, it is an extremely potent factor, since in the rotation ending with this crop, in spite of the fact that it was immediately preceded by timothy (the most detrimental of the crops tested), the corn following this rotation was the best of any of the five tested.

Since it has been shown that the roots of soybeans growing in artificially inoculated soil are as susceptible to invasion by corn root rot pathogens as those of timothy, the results of these experiments seem to indicate that some factor other than parasitism must be involved in the beneficial or detrimental effects derived from the various crops tested.

Although all experiments were conducted under greenhouse conditions and it is fully appreciated that the results obtained may be abnormal to a degree, nevertheless it seems reasonable to assume that a similar though perhaps less pronounced effect might be expected when these same crops are grown in the field.

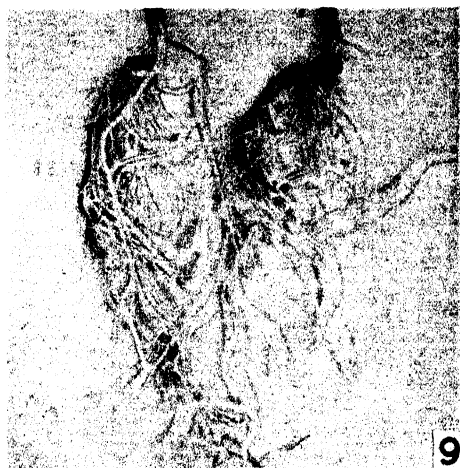
FIG. 6. Root systems of corn plants after 10 weeks' growth in root rot soil (left) steam sterilized, (right) not steamed.

FIGS. 7 and 8. Corn seedlings with roots destroyed by *P. arrhenomanes*.

FIG. 9. Root lesions caused by *P. de Baryanum*.

FIG. 10. Typical root lesions caused by *Helminthosporium* spp.

FIG. 11. Corn roots infected by *Fusarium* spp. showing slight discoloration, laterals absent on infected areas.



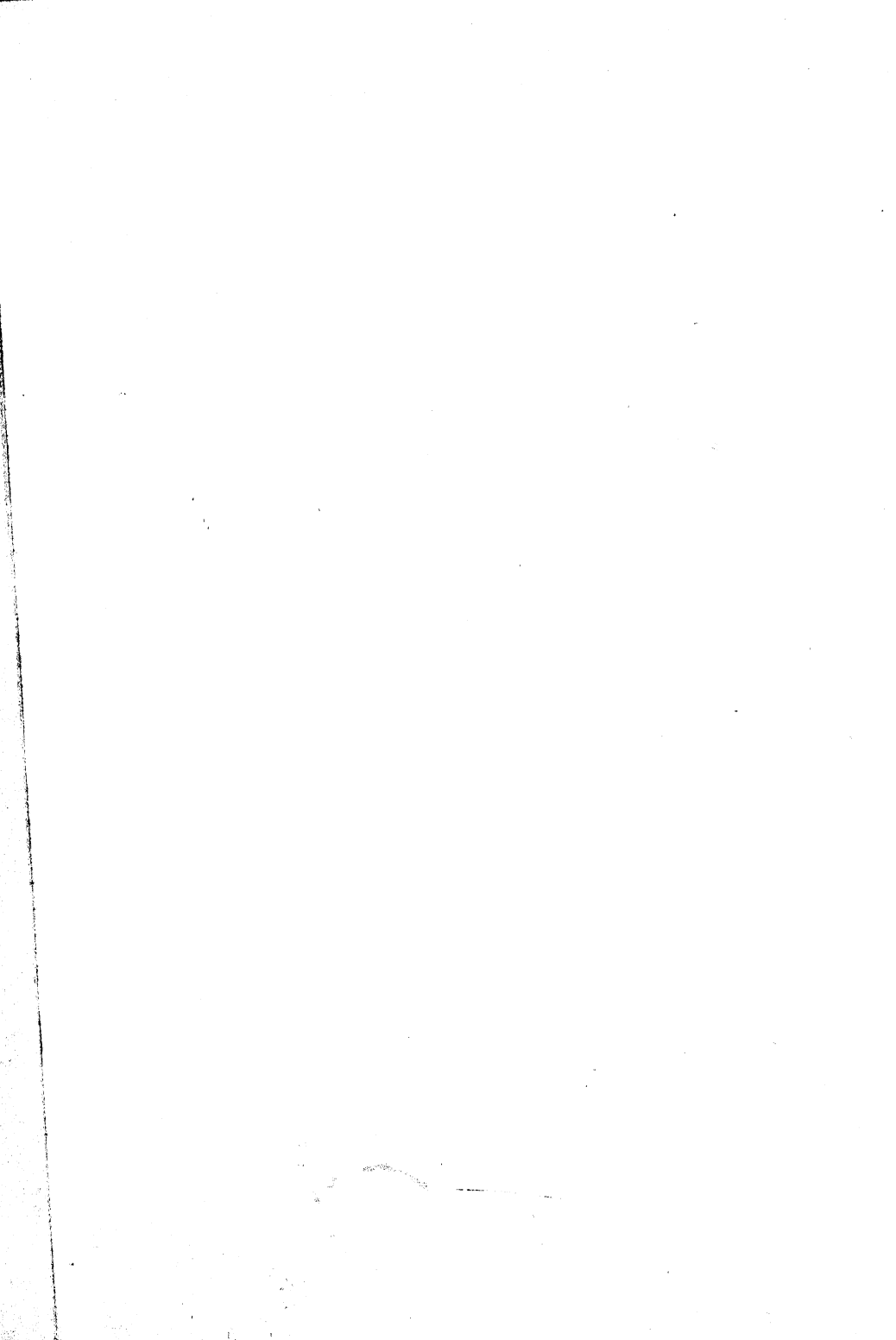


TABLE III

THE EFFECT OF ROOT ROT PARASITES ON CORN GROWING AT CONTROLLED SOIL TEMPERATURES AND WATER HOLDING CAPACITIES

Temp., °C.	W.H.C., %	Inoculum	Germination			Growth in four weeks	
			Began, days	Ended, days	%	Leaf length, in.	Wt. of 100 plants, oz.
9 to 11	60	Control	10	20	96	5.0	1.6
		Sterile medium	10	21	96	4.5	1.6
		<i>Fusarium</i>	10	19	96	5.0	1.5
		<i>Pythium</i>	—	—	0	—	—
		<i>Helminthosporium</i>	11	23	28	Died	—
	90	Control	10	19	92	3.5	1.1
		Sterile medium	10	16	84	3.0	0.9
		<i>Fusarium</i>	11	22	94	4.0	1.2
		<i>Pythium</i>	—	—	0	—	—
		<i>Helminthosporium</i>	11	17	14	Died	—
13 to 15	60	Control	6	11	96	13.0	8.6
		Sterile medium	7	13	100	11.0	6.0
		<i>Fusarium</i>	7	12	98	12.0	7.1
		<i>Pythium</i>	8	11	4	2.8	—
		<i>Helminthosporium</i>	7	13	76	3.0	0.09
	90	Control	7	12	100	16.0	10.0
		Sterile medium	6	13	100	11.0	6.0
		<i>Fusarium</i>	6	14	86	16.0	8.8
		<i>Pythium</i>	8	15	4	2.0	—
		<i>Helminthosporium</i>	6	14	68	5.0	1.9
17 to 19	60	Control	5	8	100	19.0	16.6
		Sterile medium	5	8	96	20.0	15.6
		<i>Fusarium</i>	5	7	98	18.0	12.2
		<i>Pythium</i>	6	12	46	5.0	3.5
		<i>Helminthosporium</i>	5	10	94	13.0	6.2
	90	Control	5	7	100	24.0	21.0
		Sterile medium	5	7	96	19.0	12.5
		<i>Fusarium</i>	5	11	90	23.0	17.1
		<i>Pythium</i>	5	9	30	6.0	3.5
		<i>Helminthosporium</i>	5	10	94	17.0	9.9
22 to 24	60	Control	4	5	96	20.0	17.7
		Sterile medium	2	6	100	20.0	13.5
		<i>Fusarium</i>	3	9	100	21.0	13.3
		<i>Pythium</i>	4	9	86	7.5	2.5
		<i>Helminthosporium</i>	4	9	98	19.0	9.3
	90	Control	3	6	100	26.0	26.0
		Sterile medium	4	5	100	24.0	19.0
		<i>Fusarium</i>	3	9	92	22.0	13.8
		<i>Pythium</i>	3	8	80	8.0	4.0
		<i>Helminthosporium</i>	3	6	100	20.0	12.2
27 to 29	60	Control	2	5	100	22.0	19.0
		Sterile medium	3	4	92	22.0	17.5
		<i>Fusarium</i>	3	5	98	22.0	13.4
		<i>Pythium</i>	3	6	96	13.0	6.6
		<i>Helminthosporium</i>	3	6	100	17.0	13.9
	90	Control	2	4	100	28.0	37.5
		Sterile medium	3	7	100	24.0	20.0
		<i>Fusarium</i>	2	8	94	21.0	18.6
		<i>Pythium</i>	3	6	92	13.0	7.2
		<i>Helminthosporium</i>	3	5	100	20.0	19.1

* W.H.C. = water holding capacity.

The Effect of Controlled Soil Temperature and Moisture on the Action of the Root Rot Pathogens

In the following soil temperature and moisture studies, steamed compost in controlled temperature tanks (14) was inoculated with species of *Pythium*, *Helminthosporium*, and *Fusarium*, isolated from corn roots, to determine the specific effect of the three types of organisms on corn growing under constant controlled soil conditions. Temperature ranges of 9° to 11° C., 13° to 15° C., 17° to 19° C., 22° to 24° C., and 27° to 29° C. were used and soil in duplicate containers at each of the temperatures was kept respectively at approximately 60 to 90% of its water holding capacity. Cultures of two species of each of the genera of pathogens were mixed with the surface four inches of soil in single containers and checked against uninoculated steamed compost and similar compost inoculated with the same sterile medium as that used in the culture of fungi. Each container was then planted with corn (Fig. 15).

The results in Table III show that at all but the lowest temperature (9° to 11° C.) high moisture increased the growth perceptibly over the corresponding series kept at the lower moisture content. Where no pathogen was involved, the percentage germination was not affected by either temperature or moisture, but its rapidity was reduced in direct proportion to the soil temperature. A distinct chlorosis was present in the plants growing at the lowest temperature (9° to 11° C.) with 90% moisture, a condition not present at any of the other temperatures.

The addition of sterilized culture medium to the soil appreciably reduced the growth of corn. This reduction was most pronounced in the moist soils and at the higher temperatures, being proportionately less in the lower temperature ranges.

Pythium spp. proved to be the most strongly pathogenic of the three genera tested, the effect on the host being reflected in extreme reduction in growth even at the highest temperature, which is the optimum for the development of the host (4). There was also an indication of pre-emergence killing of the seedlings evident even at the highest temperature range. This was more pronounced in the moister soils and was increasingly severe with the lowering

FIG. 12. Corn growing in root rot soil following four cover crops of (1) wheat, (2) oats, (3) rye, (4) timothy, (5) soybeans, (6) red clover.

FIG. 13. Same as Fig. 12, following (1) alfalfa, (2) rape, (3) buckwheat, (4) sugar beets, (5) tomatoes, (6) corn.

FIG. 14. Corn growing on root rot soil following crop rotations of:—

(1) buckwheat, red clover, timothy, wheat;

(2) oats, soybeans, rape, alfalfa;

(3) soybeans, wheat, alfalfa, sugar beets;

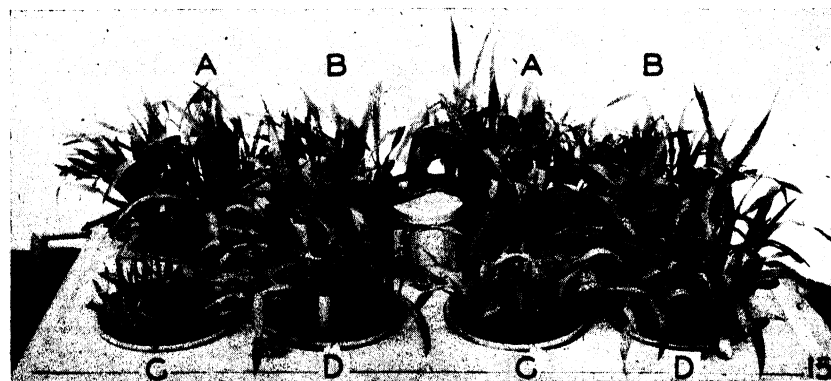
(4) red clover, buckwheat, oats, timothy;

(5) rape, rye, timothy, soybeans.

FIG. 15. Corn 14 days after planting at constant soil temperature of 22° to 24° C., not inoculated, A; inoculated with *Fusarium* spp., B; *Pythium* spp., C; and *Helminthosporium* spp., D. The four containers at left were kept at 60% water holding capacity, those at right, 90%.

of the temperature range. Many of the leaves also showed considerable malformation and failed to unfold.

The general effect of inoculum of *Helminthosporium* spp. on the subsequent crop of corn was similar to, though less severe than that of *Pythium* spp.



There was no appreciable pre-emergence killing of the seedlings except at the two lowest temperature ranges.

The detrimental effect on the corn growing in soil inoculated with *Fusarium* spp. was much less marked than with either of the other fungi. Regardless of temperature or moisture there was no reduction in germination and only a slight reduction in growth.

In each series the leaf growth coincided closely with the root development, the amount of parasitism being definitely reflected in the vigour of the plants. The particular type of injury, however, varied with the parasite involved. In roots invaded by *Pythium* infection usually commenced at the apex of the root and progressed rapidly as a light coloured, watery soft rot, destroying first the cortical tissues and later the vascular system. All roots, regardless of size, appeared subject to infection, but unless the seed was destroyed, the mesocotyl was seldom affected (Figs. 7 and 8). Roots infected by *Helminthosporium* spp. were dark brown or black and leathery in consistency. The scutellum and endosperm as well as the mesocotyl were usually infected as were most of the radicles and crown roots. The laterals on the infected areas seldom survived, but owing to the fact that the stele was not destroyed, the roots beyond the infected areas apparently functioned quite normally and sustained plant growth (Fig. 10). Quite frequently the infection travelled upwards and involved the coleoptile and older leaves. *Fusarium* spp. had a definite, though considerably less pronounced, parasitic effect resulting in the destruction of many laterals and occasionally causing light brown lesions on the larger roots (Fig. 11). In contrast with the injury caused by *Pythium* spp. and *Helminthosporium* spp., that induced by *Fusarium* spp. was more severe at the higher temperatures where the soil moisture content was low, and was practically negligible at the lower ranges.

With the exception of a few isolated contaminations it was found that the pathogens present in the corn roots were those that had been incorporated with the soil as inoculum.

Discussion

Regarding the organisms mentioned in this paper as being associated with root rot of corn, *Pythium arrhenomanes* has been described elsewhere (6, 18, 20) as a strong parasite affecting sugar-cane as well as corn. *Pythium de Baryanum*, although long considered a soil inhabiting parasite and held responsible for severe infections of many plants, has just recently been associated with root rot of corn by Wen-Chun Ho and Melhus (8), Melhus *et al.* (17), and Wen-Chun Ho (7). These investigators also mention having isolated *Helminthosporium sativum* and *Fusarium* spp. from roots of corn, but consider them of secondary importance. The observations made during the progress of the present investigation, however, leave little doubt that in addition to *P. arrhenomanes*, members of the genus *Helminthosporium* and to a lesser degree *P. de Baryanum* may be important factors in the corn root rot problem in Ontario.

Although organisms other than those dealt with in this paper may have a bearing on the corn root rot problem, the foregoing experiments prove that the organisms herein considered have definite parasitic capabilities and are the ones most frequently associated with the disease in Ontario. This, coupled with the fact that sterilized soil produces root rot free plants, suggests that regardless of the other conditions that may be involved, parasitic organisms play an important role in root rot of corn.

Since typical root rot symptoms have been produced in corn growing on different types of soil from various localities, it seems likely that with the advent of favourable environmental conditions the disease might be of significant economic importance in these areas.

While microscopic examinations of the roots of many species of plants growing in infected soil reveal a similarity in the types of organisms invading the tissues, there is a definite variation in the amount of infection and extent of root injury produced. Whether the growth of certain crops alters the balance of micro-organisms in the soil and reduces the quantity of parasitic forms present, or causes some chemical change in the soil unfavourable to the development of certain harmful organisms, the fact remains that in root rot soil in the greenhouse, corn following some crops in a rotation, notably soybeans, is practically free of root rot.

There is little doubt that soil temperature and moisture have a direct bearing on the corn root rot problem, since cool moist conditions which retard the germination of corn seed are favourable for the development of several strong root rot parasites.

Acknowledgments

Sincere thanks and appreciation are tendered Dr. G. H. Berkeley for advice and criticism during the progress of this investigation, and to Drs. J. E. Machacek and T. C. Vanterpool for their identification of fungi.

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THE STRUCTURE AND DEVELOPMENT OF THE CHROMOSOME SPIRALS IN MICROSPORES OF *TRILLIUM*¹

BY A. H. SPARROW²

Abstract

The structure and development of somatic spirals in microspore chromosomes of *Trillium* have been investigated. The chromonemata in each metaphase chromatid and each anaphase chromosome form a large-gyred, hollow spiral. This spiral develops gradually during prophase by an increase in gyre diameter and a decrease in gyre number and in chromatid length. Its development is associated with the *elimination* not the *production* of chromatid relational coiling. At later stages an irregular waviness or "minor" somatic spiral is visible along its "major" gyres in which reversals of direction can also be discerned. Where the spiral can be seen to be double-stranded it is plectonemic (as early as mid-prophase).

The prophase to metaphase chromatid contraction ratio is not less than 6 : 1. Mean chromonema length increases from 650 ± 17.2 at metaphase to 977 ± 28.3 at anaphase. This latter length is approximately that estimated for early meiotic prophase. Chromosome volume also increases (about twofold) during the interval between metaphase and anaphase. Mean chromonema length and gyre number in microspore anaphase chromosomes are more than twice as great as those of meiotic anaphase chromosomes. Since the chromosomes at these stages are of approximately the same mean length the gyres of the somatic spiral are thus more tightly "packed".

In *Trillium*, microspore anaphase chromosomes are considered to be of essentially the same spiral structure as meiotic second division chromosomes, i.e., a single coil (but *not* single-stranded), rather than two or more independently coiled chromatids. The process of reducing this plectonemic spiral into parallel, freely-separable chromatids begins in one prophase as a reduction in gyre number and continues as relational uncoiling in the next. Paradoxically, therefore, a spiralization cycle such as that described above can be interpreted as an uncoiling process in which successive cycles overlap in prophase.

Introduction

As a prerequisite to any study of chromosome mechanics a thorough grasp of their micromorphological features throughout all stages of the mitotic cycle is of paramount importance. Unfortunately, previous investigators have differed widely in their interpretations of somatic chromosome structure and naturally those who have considered the mechanics of spiralization have formulated theories almost as diverse as their interpretations of structure.

It is now generally recognized by cytologists that the chromonemata of somatic chromosomes are spiralized to a variable degree at all stages of mitosis (e.g., 2). The disagreement arises as to the number of spirals, their structure, interrelationships, and ontogeny. Different teleological interpretations are also given. The present investigation of mitotic chromosomes of *Trillium* was initiated in the hope of clarifying at least some of these important problems, with the ultimate goal of a single unified hypothesis to explain both somatic and meiotic coiling.

¹ Manuscript received September 22, 1942.

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A clear understanding of the spiral structure of somatic chromosomes has lagged considerably behind the elucidation of meiotic chromosome structure. There is no dearth in the early literature of descriptions or illustrations of telophase and prophase spirals, but the relatively rare observations of metaphase or anaphase spirals led to the conclusion that the appearance of spiral structure was the exception rather than the rule (44). It is now apparent that one of the most important factors responsible for this unfortunate situation is that techniques which successfully demonstrate spiral structure in meiotic chromosomes usually show relatively little spiral structure at somatic metaphase and anaphase. The greater chromaticity of the matrix, as well as the closer packing of the gyres of somatic chromosomes, is, presumably, involved in determining this characteristic difference. Much confusion has also arisen from observations of chromosomes showing a high degree of vacuolation. Such a structure is now generally considered to be an artifact resulting from poor fixation (14, 20, 31).

The spiral structure of mitotic anaphase chromosomes or metaphase chromatids has been variously interpreted by different investigators to fall within one of the following categories: (i) a single spiral, (ii) two non-intertwining threads, (iii) two intertwining threads, or, (iv) two or more independently spirialized threads that may be more or less twisted about each other¹. According to some interpretations these categories overlap somewhat, as in the case of early anaphase chromatids being parallel but becoming progressively twisted about each other at later stages (1). Also it is considered by some investigators that an apparently single-stranded spiral may actually be composed of two or more closely appressed chromonemata that may not appear visibly distinct until telophase or the following prophase (58, 18, 20, 53, 1, 43, 7, 8). The rather contradictory views in the literature on the number of chromonemata Kuwada (38) explains "by the assumption that under certain circumstances the four chromonemata appear by union to be two or even one." This may account for contrasting reports of double-stranded vs. single-stranded spirals, but it is rather difficult to imagine four, or even two, independently spirialized chromonemata suddenly merging into one optically single-stranded spiral.

In accord with the greater attention paid to meiotic than somatic spirals, theories of spiralization have been based largely on such studies. The various theories so far formulated may be roughly divided into two groups, those in

¹ The investigators upon whose work this classification is based include the following: (i) Kuwada, 1926 (36); Geilker, 1935 (18), 1938 (19, 20); Sax and Sax, 1935 (53); Gustafsson, 1936 (22); Upcott, 1936 (64); Darlington, 1937 (13); Mather, 1937 (42); White, 1937 (67); Darlington and Upcott, 1939 (15), 1941 (16); Manton, 1939 (40); Coleman, 1940 (7, 8); Hillary, 1940 (24); (ii) Sharp, 1929 (57); Straub, 1938 (61); (iii) Brunelli, 1910 (6); Schneider, 1910 (56); Dehorne, 1911 (17); Kaufmann, 1926 (31, 32); Telserynski, 1930 (62), 1931 (63); Hedayatullah, 1931 (23); Hsu-Siang, 1932 (26); Perry, 1932 (52); Smith, 1932 (58); Kosky, 1933 (34), 1937 (35); Hoare, 1934 (25); Marshak, 1936 (41); Jeffrey, 1937 (30); Naikani, 1937 (45, 46); Atwood, 1937 (4); Warmke, 1937 (66); Wolcott, 1937 (69); Creighton, 1938 (9); Mensinkai, 1939 (43); (iv) Nebel, 1933 (47); Goodspeed, Uber, and Avery, 1935 (21); Stebbins, 1935 (60); Nebel and Ruttle, 1936 (50); Kuwada, 1939 (38); Kuwada and Nakamura, 1940 (39); Aisima, 1941 (2).

which the matrix is regarded as the dynamic force with the chromonema playing a minor role (3, 54, 55) and those in which changes within the chromonema are of paramount importance (36, 37, 5, 11, 27, 48, 68). These theories cannot be discussed in detail here. Suffice it to say that they include such mechanisms as contraction of the matrix, elongation of the chromonema, anisotropic swelling, molecular reorientation, heterogonic growth, pH changes, etc. Obviously many of these postulated mechanisms cannot be tested experimentally, but two factors are now known definitely to be associated with the formation of the major coil of meiosis. They are shortening of the matrix (in *Tradescantia*) and elongation of the chromonema (in *Trillium*). In mitosis it is known that the development of the new somatic spiral in each prophase is associated with chromatid (hence matrix?) contraction, but so far no one has measured chromonema length changes during these stages.

Somatic spirals can be seen to increase in diameter from early prophase to metaphase and still further in their expansion into relic coils. However, it is not agreed whether or not the gyre number decreases as their size increases. Husted (29), Koller (33), and Abraham (1) consider that they do decrease in number, while Nebel (49) holds the opinion that the prophase gyres are "not much, if any, more numerous than the final standard gyres."

Materials and Methods

Observations were made on microspore chromosomes of two species of *Trillium*. The data presented herein are from *T. grandiflorum*. Less extensive observations have also been made on *T. erectum*. The best preparations for internal structure were smears stained in iron-aceto-carmin. After replacement with 45% acetic acid and sealing, the slides were kept in a refrigerator until used. The internal structure seemed clearer in old slides than in material freshly prepared. Some of these "temporary" slides are still in good condition more than a year after their preparation.

The methods used in making measurements have been described by Sparrow, Huskins, and Wilson (59) and will not be repeated in detail here, except to give the formula used in calculating the length (L) of a spiralled thread:

$$L = n\sqrt{p^2 + (\pi d)^2}$$

when n = number of gyres, p = pitch, and d = diameter of the gyre minus the diameter of the chromonema. All lengths given are totals for a set of five chromosomes.

Observations

Morphologically, chromatid contraction is the most obvious change that chromosomes undergo during prophase. This contraction is accompanied by two processes which at first sight seem antithetic: (1) the unwinding of the (relic) spirals carried over from the previous anaphase, and (2) the development of a new somatic spiral within each chromatid. In a previous paper the unwinding of the relic coil of meiosis and the origin and elimination of chromatid relational coiling in the microspore prophase have been considered (59).

The study has now been extended to include the accompanying structural changes within the chromatids, i.e., the structure and development of the somatic spiral from early prophase through metaphase and anaphase of the first division of the microspore. Two illustrations of spiral structure at prophase of the following division (i.e. pollen tube) are also presented.

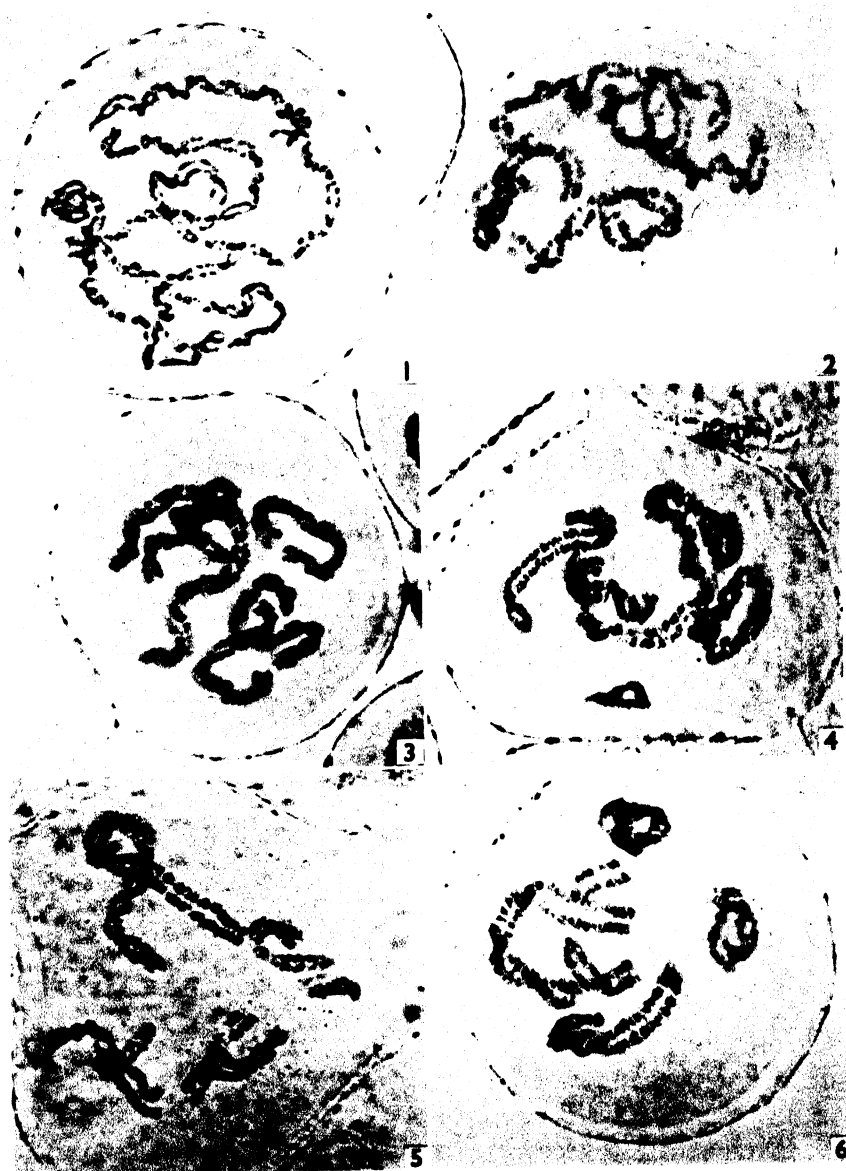
Chromatid lengths in 10 microspore prophase nuclei varied between 410 and 590 μ , with a mean of $467 \pm 17 \mu$. The high variation is, presumably, due to the fact that prophase contraction has proceeded further before fixation in some nuclei than in others. The above mean is therefore regarded as an underestimate of early prophase length. A length of at least 500 μ would be a conservative estimate of the initial length, 600 μ would not be unexpected. Mean metaphase chromatid length measured in 15 cells is 90.5 ± 3.9 . These figures give a contraction ratio of about 6 : 1 between early prophase and metaphase. No significant change occurs in chromatid length between metaphase and anaphase, but mean chromatid diameter increases significantly. The total increase in chromatid diameter between early prophase and anaphase is more than threefold (from less than one to almost three micra).

The relic spirals of microspore prophase are derived from the meiotic major spiral. Likewise it seems that the waviness visible along the gyres of the relic coil corresponds to the "minor spiral" of meiosis. This has been checked by counting the number of "waves" and "minor gyres" at the two stages. Their number is estimated to be between 480 to 600 for a coil of 60 gyres, i.e., about 8 to 10 per gyre of the major or relic coil. During early prophase the irregularly waved chromonemata within the chromatids gradually assume the form of a regular spiral. The growth of this spiral by an increase in gyre diameter and a reduction in gyre number is illustrated in Figs. 1 to 8. Chromatid lengths and gyre number at various stages from early prophase to anaphase are given in Table I. As the number of gyres in prophase stages cannot readily be determined by direct counting throughout whole complements the total number of gyres per complement has been estimated from counts made

TABLE I

CHROMATID LENGTH (IN MICRA) AND GYRE NUMBER AT PROPHASE, METAPHASE, AND ANAPHASE STAGES IN MICROSPORES OF *T. grandiflorum*

Stage	Chromatid length	No. of gyres
Relic coil of 60 gyres	450-600	480-600
Prophase (Fig. 1)	346	554
Mid-prophase (Fig. 2)	202	242
Mid-prophase (Fig. 3)	205	276
Later prophase (Fig. 4)	173	151
Later prophase (Fig. 5)	154	170
Later prophase (Fig. 6)	142	187
Metaphase (Fig. 8)	77	130
Metaphase (Mean of 15 cells)	90.5 ± 3.9	120 ± 5.1
Anaphase (Mean for 15 cells)	95.0 ± 2.9	130 ± 3.3



Prophase stages showing progressive changes in somatic and relational coiling in microspores of *T. grandiflorum*. Magnification, 1000 \times . From temporary aceto-carminc smears.

FIG. 1. Prophase (346 μ and 554 gyres). FIGS. 2 AND 3. Mid-prophase (202, 205 μ and 242, 276 gyres, respectively). FIGS. 4, 5, AND 6. Later prophase stages (173, 154, 142 μ and 151, 170, and 187 gyres, respectively).



Spiral structure in metaphase and anaphase of microspore mitoses and relic coils in pollen tube prophase nuclei. FIGS. 7 TO 12, *T. grandiflorum* (a and c of Fig. 10 are different prints made from the same negative, b is taken at a slightly different focus); FIGS. 13 AND 14, *T. erectum*. Magnification, 1000 \times . From temporary aceto-carminic smears.

FIGS. 7 AND 8. Metaphases. FIGS. 9, 10, 11, AND 12. Anaphases. In Figs. 7 and 10 the optical cross sections show the irregularly waved chromonemata and the hollow spiral (See text, p. 261). FIGS. 13 AND 14. Relic coils in pollen tube prophase nuclei of *T. erectum*.

in regions where the gyres are most clearly visible. Lengths in these regions were obtained from measurements of camera lucida drawings which were frequently checked from photographs or with micrometers.

Measurements of metaphase and anaphase chromonema lengths have been made in five cells at each stage in three different plants (Table II). The mean gyre diameter, gyre length, and total chromonema length all increase significantly. Mean chromonema length for 15 cells increases from 650 ± 17.2 at metaphase to 977 ± 28.3 at anaphase. The latter length is approximately that of the early meiotic prophase of *T. erectum* (59). Chromosome volumes have not been given in the table but calculations made from the data presented indicate that the mean volume at anaphase is about double that at metaphase.

TABLE II

MEASUREMENTS AT METAPHASE AND ANAPHASE OF SOMATIC SPIRALS IN *T. grandiflorum* MICROSPORE COMPLEMENTS

Plant number	No. of cells	Stage	Gyre diam.	Chromosome length	No. of gyres	Chromonema length	Mean gyre length
69-G-92-G	5	M	2.34 ± 0.245	100 ± 7.7	104 ± 3.1	602 ± 46.8	6.57 ± 0.45
	5	A	3.10 ± 0.185	101 ± 2.9	118 ± 6.6	1002 ± 27.9	8.82 ± 0.53
69-G-100-G	5	M	1.95 ± 0.131	83 ± 4.7	136 ± 11.3	682 ± 6.1	5.16 ± 0.44
	5	A	2.54 ± 0.072	89 ± 7.0	137 ± 4.0	917 ± 27.3	6.66 ± 0.26
69-G-104-A	5	M	1.98 ± 0.087	88 ± 5.2	121 ± 1.9	644 ± 21.5	5.32 ± 0.27
	5	A	2.68 ± 0.133	94 ± 3.8	134 ± 2.4	1011 ± 39.9	7.53 ± 0.42
69-G-92,-100, and -104	15	M	1.95 ± 0.097	90.5 ± 3.9	120 ± 5.1	650 ± 17.2	5.68 ± 0.27
	15	A	2.77 ± 0.099	95 ± 2.9	130 ± 3.3	977 ± 28.3	7.67 ± 0.30

Metaphase and anaphase microspore chromosomes have essentially the same structure as those of the second meiotic division, i.e., a single double-stranded spiral. The gyres are, however, more numerous, closer together, and hence more difficult to distinguish. The somatic spiral is composed of two visibly distinct chromonemata, which, like the half-chromatids of meiosis, are irregularly waved. This waviness can be seen in optical cross sections (Figs. 7, 10) as well as in side views (Fig. 11) and in neither case does it approach the form of a regular spiral. In certain selected regions the two chromonemata can be seen to be wound in the form of a plectonemic spiral even as early as mid-prophase. There seems to be considerable variation as to the closeness of the two associated chromonemata. In some regions they are quite widely separated and in others even within the same chromosome they appear as a single-stranded spiral. Changes of direction have also been observed but their frequency has not been determined.

The chromonemata of metaphase and anaphase chromosomes occupy only the peripheral region of the chromosome. There is a central core of lower chromaticity. This is best seen in optical cross sections (Figs. 7, 10), but can

also be seen in longitudinal optical sections (Fig. 7, right arm of lower chromosome). The latter is considered by the author to explain the frequent claims in the literature that anaphase chromosomes are composed of two independently spiralized chromatids (2). With a slight change of focus these so-called "independently spiralized chromatids" mysteriously disappear to give cross-striations extending right across the chromosome (Figs. 8, 9). The latter represent the true gyres. The former are optical illusions.

Preliminary observations of pollen tube mitoses have been made in regard to chromosome structure and mechanics. The results obtained essentially confirm those obtained from studies of microspore mitosis. Here again, relational coiling is derived from plectonemic relic coils (Figs. 13, 14) and the somatic spirals apparently "grow up" by an increase in gyre diameter and a decrease in number.

Observations have also been made on meiotic spirals in *Tradescantia* by Dr. S. G. Smith. He considers that the minor spiral present at first anaphase (which is a more regular spiral than the corresponding waviness in *Trillium*) develops into the typical second anaphase spirals. The major coil of first division is eliminated at the prophase of the second division in *Tradescantia* while it persists in *Trillium* until microspore prophase. In the latter case the second division occurs without any intervening interkinetic resting stage and is not accompanied by a new spiralization. This is of especial interest in that it shows that spiralization does not always keep pace with the spindle mechanism. In the absence of an interkinetic resting stage in *Trillium* the new spiralization does not occur until microspore prophase, but the activity of the spindle is, to all appearances, normal. A comparative physiological study of meiosis in species with and without interkinesis might throw some light on the changes determining nuclear or chromosomal transformations as distinct from those associated with spindle activity.

Discussion

The value to theoretical cytology of accurate measurements of such obviously important factors in the chromosome spiralization cycle as size changes cannot be overestimated. Unfortunately this has been recognized by only a few cytologists, e.g., Wilson and Huskins (68), Manton (40), and Darlington and Upcott (15). Meiotic changes especially at prophase have been studied by a number of workers (see summary, by Darlington and Upcott, 15) but mitotic stages have been almost neglected. Prophase chromatid contraction has been measured by Sax and Sax (53) and by Patau (51). The former found a prophase to metaphase ratio of 5 : 1 in *Tradescantia* microspores and the latter, 5.5 : 1 in *Collozoum*. These ratios agree very closely with the 6 : 1 ratio for *Trillium* microspores reported here.

As far as the author is aware, Manton (40) is the only worker who has measured lengths of spiralled chromonemata in mitotic chromosomes. From her work on *Osmunda* she states that "the real length of the chromosome thread is thought to be constant throughout the mitotic cycle."

Bridges (3), and Naithani (46) are also of the opinion that the gene thread or chromonema maintains a constant length. The present data which show an elongation of the chromonema between metaphase and anaphase in microspore chromosomes of *Trillium* are not in accord with the opinions of the above workers, for an increase as great as 327μ (more than nine times the standard error) can hardly be due to chance variations either in absolute lengths or in errors of measurements. Nor can the increase be attributed to the straightening out of a "minor spiral" for the irregular waviness at anaphase appears more copious than at metaphase.

Darlington and Upcott (15) have abstracted some of the published work on chromosome and chromonema size. They find that the measurements of "several workers" agree in giving what they call a "packing factor" $\frac{D}{d}$ of 2.0 to 2.5. (" D = diameter of chromatid, d = diameter of chromosome thread or of the region occupied by it".) As "probably most compatible with observation and with the model" their illustrations of chromosomes representing this value are solid spirals. The observations on *Trillium* spirals are not in accord with this conclusion as both meiotic major and somatic spirals are clearly seen to be hollow. Chromosomes in fresh pollen mother cells of *Trillium* mounted in 4% cane sugar solution also show a hollow spiral structure according to Wilson (unpublished data).

It has been stated above that the somatic spiral is considered to grow up by an increase in gyre size and decrease in gyre number. In tests with models the expanding gyres proximal to the attachment get their extra length at the expense of the distal ones. Since gyre diameter seems to be fairly uniform for a chromosome arm, at a given stage, but increases gradually over a period of time the expansion per gyre will be proportional to the number lost at the end. Hence each time a gyre is lost the redistribution must extend all down the line. Redistribution can, however, occur from changes of direction as well as from the end. It seems probable, therefore, that reversals may play a part in the development of the somatic spiral as well as in untwisting of prophase chromatid relational coiling as previously described (59).

It is the opinion of the writer that the pairs of chromonemata separated by the quaternary split (the split that becomes effective at anaphase of pollen tube mitosis) are plectonemic at mid-prophase of the first microspore mitosis. They may also be plectonemic (or relationally coiled) at earlier prophase stages before their relationships can be determined by optical methods. In this connection it should be borne in mind that the time at which the plane of the split is determined is at least as important, if not more important, than the time at which it becomes visible. Obviously information on the former point is beyond hope of attainment with present techniques, and one can do no more than speculate as to how and when the plectonemic condition arises. If it were to arise after the initiation of the somatic spiral the split would necessarily be a spiral cleavage in the same direction as the gyres of the spiral. If the plane were already present or determined before the chromonemata

became regularly coiled the attainment of the plectonemic condition might require that the chromonemata twist about each other—or they might untwist, this depending upon the original amount of twisting present.

Several authors have suggested that in mitosis the daughter chromatids are twisted about each other at the time of their inception (31, 35, 46, 38). This seems not unreasonable and is in accord with their observed plectonemic condition at mid-prophase in *Trillium* microspores. Before the sister threads in this plectonemic coil can separate at the anaphase of the following division they must undergo extensive unwinding. Further, the amount of unwinding required is proportional to the number of gyres present. Therefore any reduction in gyre number of a plectonemic coil is the equivalent of untwisting in the derived relational coil. Hence the untwisting or unwinding process necessitated by the fact that the somatic coil is plectonemic begins with the decrease in gyre number in one prophase, continues in the further reduction during the transition to the relic coil of the next prophase and is completed with the final elimination of the twists in the chromatid relational coil derived from the plectonemic relic.

In the above interpretation prophase chromatid untwisting is a continuation of the changes initiated in the previous division and not a reversal (*contra* Abraham, 1). Concurrent with this untwisting a new spiral is developing within each chromatid so that successive cycles overlap in prophase. But, paradoxically, even the further development of this new spiral is an unwinding process in that any reduction of gyres is the equivalent of an elimination of relational twisting. In the material studied there has been found no sudden reversal of the process of untwisting to one of retwisting (cf. 38, 1) nor any evidence of chromatids actively twisting about each other during prophase as frequently suggested (12, 28, 65, 10). The growth of the new internal spiral seems to be associated with the *elimination* of relational twisting and not with its *production*, as suggested by some of the authors referred to above. Further, if a process of active twisting is to be postulated to account for the plectonemic condition, it must occur within each chromatid one division in advance of that at which it is finally undone and at a very early stage before the fine threads can be observed with sufficient resolution to detect it. The amount of twisting necessary on this hypothesis to give the observed number of plectonemic gyres would seem to be prohibitive. It is therefore tentatively suggested that the twisted condition that presumably prefaces plectonemic coiling may be structurally inherent rather than the result of torsion affecting the pairs of chromonemata or chromatids after their dual nature can be optically resolved.

Acknowledgments

The author wishes to thank Professor C. L. Huskins and Dr. G. B. Wilson for their interest in the problem and reading of the manuscript.

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CHROMOSOME BEHAVIOUR AND FERTILITY IN DIPLOID WHEAT WITH TRANSLOCATION COMPLEXES OF FOUR AND SIX CHROMOSOMES¹

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Abstract

Seven different reciprocal chromosome translocations involving six of the seven haploid chromosomes of *Triticum monococcum* have been studied in all possible heterozygous combinations. Plants with one complex of four chromosomes show only 5 to 10% sterility; those with two complexes of four, 10 to 20%; those with one complex of six, 20 to 30%. Completely random segregation in such types should cause 66.6, 88.8, and 90% sterility, respectively, while segregation directed only by the necessity that homologous centromeres go to opposite poles should cause 50, 75, and 75%, respectively. The very low sterility of translocation heterozygotes in wheat, as compared with these expectations, and with results reported in maize and other plants, is due to the fact that segregation is usually alternate (disjunctional) in complexes both of four and six.

The forces of repulsion operating at metaphase are not restricted to the centromere but involve the whole body of the chromosome. In the absence of complicating features this naturally results in alternate segregation in complexes. Semisterility is due, not to basically random segregation, but to special conditions such as interstitial chiasmata, early opening of the complex, and crossing over between the centromere and the point of interchange. The latter is favoured by a non-median position of the centromere, great length of chromosomes, and shortness of at least one interchanged segment. The 50% sterility usually reported for translocations has no special significance; no particular percentage is characteristic of translocations in general.

In complexes of six, double-cross configurations are more numerous than stars, and present many variations in form depending on the length and position of the segments exchanged. Additional factors producing sterility in large complexes are unwieldiness and crossing over in the segment that joins the two crosses.

Introduction

Plants that are heterozygous for reciprocal chromosome translocations show, in different cases, very different degrees of sterility. The impression that as a rule they are semisterile is apparently due to the fact that much of the work on translocations, and particularly the early work, has been carried on with maize, in which nearly all are reported to involve sterility of 50%. In fact, maize plants that are heterozygous for a translocation are commonly referred to as "semisteriles". The situation in peas is similar. But several examples in wheat were shown to be almost fully fertile (13, 14). Nearly complete fertility appears to be the rule also in *Datura*. In other genera, such as *Tradescantia* and *Campanula*, although sterility has been reported, it may be much less than 50%.

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The causes of these differences in sterility must be bound up with the causes of the sterility itself. The reason always given for the situation in maize is that random two-by-two segregation takes place in the translocation complex of four chromosomes. When any two chromosomes that are adjacent in the ring go to the same pole the resulting spore always has both a deficiency and a duplication and is therefore inviable (Fig. 1, F to I), whereas when alternate chromosomes go together (Fig. 1, D and E) the resulting spore always has the normal chromatin complement (either in the original or in the new arrangement) and is therefore fully viable. It has been assumed that "alternate" and "adjacent" segregations are equally frequent and hence that the sterility is 50%. But in true random segregation two-thirds of the spores (not one-half) receive adjacent chromosomes, as is demonstrated in Fig. 1. Consequently random segregation should result in 66% sterility, not the reported 50%.

The percentage of incompetent spores would be reduced from 66 to the observed 50% if two chromosomes whose centromeres are paired in prophase (homologous) must always go to opposite poles. In that case the two combinations *F* and *I* of Fig. 1 would not be formed. But since the complex opens out into a ring before segregation there is no apparent reason why homologous centromeres should go to opposite poles. Furthermore McClintock (9) has shown that in maize they actually may go to the same pole.

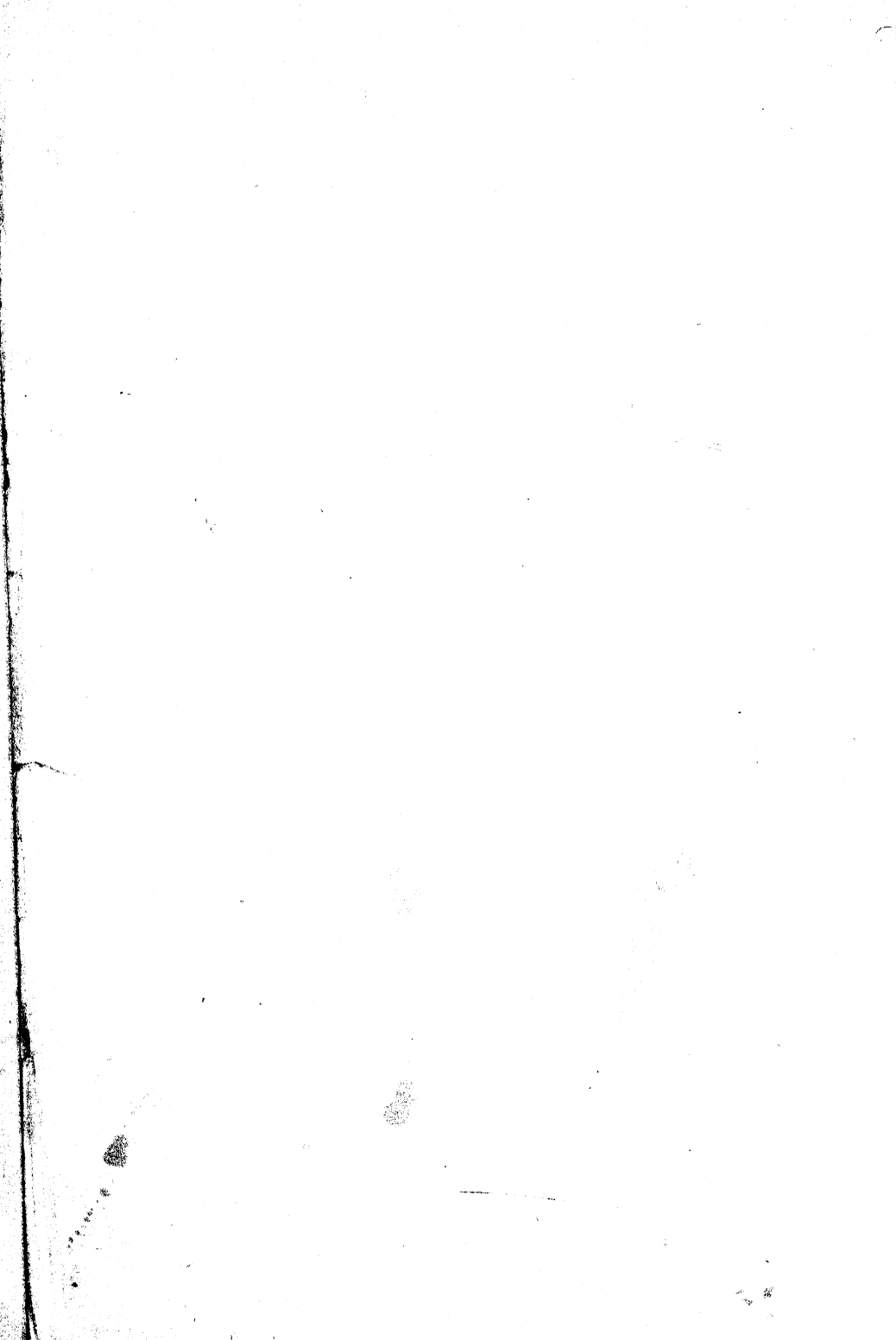
The reason that fertility is nearly complete in the wheat plants studied is that segregation from the complex of four chromosomes is regularly alternate, according to Thompson and Thompson (14). At metaphase the complex regularly takes the form of a horizontal figure of eight. Smith (12, 13) did not observe this directed segregation and was therefore at a loss to account for the high fertility. There can be no question that, in the material studied in the present investigation, the segregation is alternate in the great majority of cases; the amount of adjacent segregation corresponds closely to the amount of sterility.

The problem therefore is to determine why segregation should be regularly alternate (disjunctional) in one genus and random in another. On general principles one would expect that the forces that cause the segregation of the chromosomes would work out in the same way in all plants. It was therefore decided to undertake the following studies: (1) an investigation of additional translocations in wheat and of hybrid combination with two complexes of four chromosomes, in order to leave no doubt as to whether disjunctional segregation, resulting in full fertility, must be regarded as the normal procedure in wheat; (2) a careful study of chromosome behaviour, particularly during prophase, in comparison with other genera, with a view to determining the reasons for the differing behaviour.

A similar set of problems arises in connection with complexes of six chromosomes. Such a complex is produced when a cross is made of two types each



FIGS. 3 TO 8. Photographs showing translocation complexes at various stages. $\times 1000$.
 FIG. 3. Pachytene stage in a nucleus with two complexes of four chromosomes and three pairs. The centres of the two cross-shaped complexes are indicated by the arrows. FIG. 4. Late prophase; two cross-shaped complexes of four (in centre) and three bivalents. FIG. 5. Metaphase; figure of eight and zigzag chain at right, three bivalents at left. FIG. 6. Prophase; star-shaped complex of six chromosomes plus four pairs. Three of the latter are towards the left and above, one at the right and below, crossing one arm of the star. FIG. 7. Metaphase; complex of six in the form of a zigzag ring, and four bivalents. FIG. 8. Metaphase; zigzag chain of six, plus four pairs.



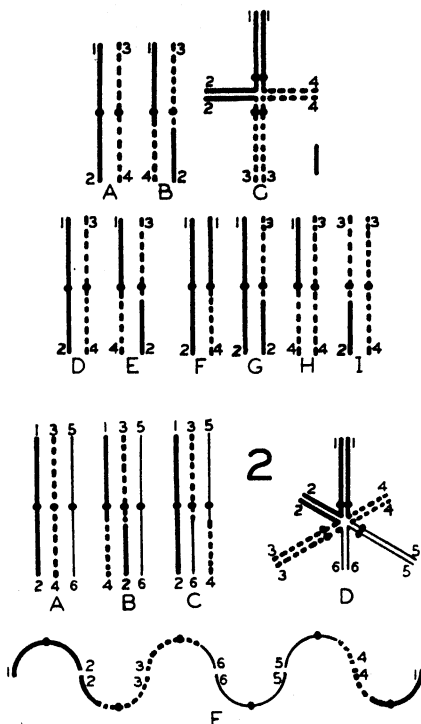


FIG. 1. A, original chromosomes; B, after reciprocal translocation; C, cross-shaped complex in meiotic prophase; D and E, chromosomes resulting from alternate segregation; F to I, chromosomes resulting from adjacent segregation.

FIG. 2. A, original chromosomes; B, after first translocation; C, after second translocation; D, star-shaped complex at prophase; E, zigzag configuration at metaphase in alternate segregation (each set of three chromosomes (going up or down) includes a full complement of chromosome material, and would result in a fully viable spore).

of which is homozygous for a different translocation involving a common chromosome. In maize such plants are reported to show 70% sterility. If segregation from the complex were always alternate only two combinations of chromosomes would be produced, as shown in Fig. 2, and both of these would be viable; consequently no sterility should occur. But if segregation is random three-by-three, 20 different combinations would occur with equal frequency. The only viable combinations would be the two that result from alternate segregation. Therefore random segregation would cause 90% sterility instead of the 70% reported for maize. If segregation were such that homologous centromeres must always go to opposite poles but is not otherwise directed, eight combinations would be formed with equal frequency, as may be seen by reference to Fig. 2. Again only two would be competent; consequently the sterility should be 75%. It was therefore decided to investigate complexes of six in the same way as complexes of four.

The Translocations and Their Relationships

Seven reciprocal translocations in *Triticum monococcum*, produced by radiation treatment, were used. These were designated T_1, T_2, \dots, T_7 . Homozygous types, which are all fully fertile and indistinguishable from the original material, were crossed in all possible combinations, and all F_1 combinations were examined cytologically to determine whether they had two complexes of four chromosomes or one of six. The results are given in Table I. From the data in this table the relationships of the chromosomes involved in the different translocations may be worked out.

TABLE I

CHROMOSOME COMPLEXES IN HYBRIDS BETWEEN LINES HOMOZYGOUS FOR DIFFERENT TRANSLOCATIONS. TWO COMPLEXES OF FOUR CHROMOSOMES ARE DESIGNATED "4", AND ONE OF SIX IS DESIGNATED "6"

Translocation number	Translocation number						
	1	2	3	4	5	6	7
1	x	6	6	6	4	4	4
2		x	4	6	4	4	6
3			x	6	6	4	6
4				x	4	4	6
5					x	6	6
6						x	4
7							x

Let the original seven haploid chromosomes of *T. monococcum* be designated 1, 2, 3, 4, 13, 14, each pair of numbers representing the two ends of a single chromosome. Let the chromosomes involved in T_1 be 1.2 and 3.4, and let the chromosomes with successively higher numbers be assigned to successively higher-numbered translocations, in so far as this is necessary to satisfy the data of Table I. The results are given in Table II. The conclusions shown in this table are throughout consistent with the data given in Table I, and are necessary in order to account for those data. Observations

TABLE II

THE CHROMOSOMES INVOLVED IN EACH OF THE TRANSLOCATIONS STUDIED

Translocations	Chromosomes						
	1.2	3.4	5.6	7.8	9.10	11.12	13.14
T_1	1.2	3.4					
T_2		3.4	5.6				
T_3	1.2			7.8			
T_4	1.2		5.6				
T_5				7.8	9.10		
T_6					9.10	11.12	
T_7			5.6	7.8			

to be reported later show that when a particular chromosome was involved in more than one translocation the segments transferred came either from the same or from the two different arms.

It will be observed that with one exception (13.14) each of the seven original chromosomes is involved in at least one translocation. Three of them are involved in three different translocations, two in two, and one in one. The study of these seven should, therefore, give a representative picture of the behaviour of translocations in wheat.

Sterility

In a previous paper (14) it was reported that plants that are heterozygous for any one of three of these same translocations and therefore have an association of four chromosomes at meiosis, show only from 5 to 10% sterility. The additional four translocations included in this study cause approximately the same amount. The data for plants that have various combinations of two translocations and therefore show either two complexes of four or one of six, are given in Table III.

TABLE III

PERCENTAGES OF STERILITY IN PLANTS HETEROZYGOUS FOR TWO DIFFERENT TRANSLOCATIONS

Complexes	Translocations involved	Female		Male	
		Total examined	Sterility, %	Total examined	Sterility, %
1 of 6	$T_1 \times T_3$	275	18.6	5042	24.5
	$T_1 \times T_4$	124	17.0	1025	20.5
	$T_2 \times T_4$	143	29.0	974	36.8
	$T_2 \times T_7$	345	22.8	220	16.4
	$T_3 \times T_4$	80	21.2	431	28.2
	$T_3 \times T_7$	174	25.1	1163	21.4
	$T_4 \times T_7$	209	24.4	1432	23.2
	$T_6 \times T_7$	302	19.5	520	24.4
	$T_6 \times T_7$	148	23.6	421	32.2
2 of 4	$T_1 \times T_6$			612	11.0
	$T_1 \times T_6$	226	13.3	556	13.0
	$T_1 \times T_7$	331	21.4	811	23.0
	$T_2 \times T_3$	175	17.1	1320	22.0
	$T_2 \times T_6$	121	22.8		
	$T_2 \times T_6$	278	11.2	1926	12.7
	$T_3 \times T_6$	220	12.7	801	21.3
	$T_4 \times T_6$	346	11.6	1321	18.5
	$T_6 \times T_7$	287	14.9	315	14.4

It will be observed that in most types with a complex of six, the sterility is between 20 and 30%, but may fall a little below or rise a little above these figures. This is far lower than the 90% that is to be expected if segregation is random and also much lower than the 75% that is to be expected if the only restriction in random segregation is that homologous centromeres must go to opposite poles. A sufficient number of specimens of the different types

were studied both on the male and female sides to prove that at least some of the differences in sterility between the different types were consistent and significant.

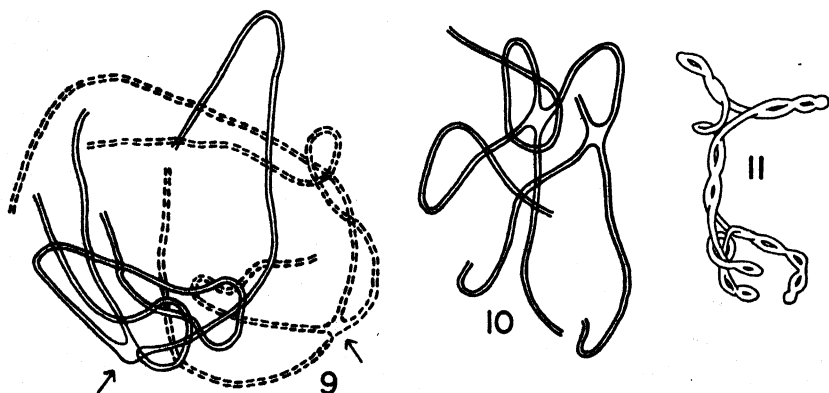
In most plants with two complexes of four chromosomes the sterility is between 10 and 20%. This again is much lower than the 88% to be expected with random segregation or the 75% to be expected if segregation is directed only to the extent that homologous centromeres must go to opposite poles. It is in good agreement with the percentages to be expected on the basis of results obtained with one complex of four.

The reason that plants with one complex of six are somewhat more sterile than those with two complexes of four, will be discussed later.

Chromosome Behaviour

IN COMPLEXES OF FOUR

All the chromosomes of *T. monococcum* are so similar in their morphological features, as first described by Kagawa (8), that only with great difficulty and much labour can they be identified separately at mitosis. It is impossible to identify them in meiotic stages. Of particular importance in relation to the fertility of translocation heterozygotes are the facts, (1) that they all have nearly median centromeres, and (2) that the chiasmata are usually terminal at metaphase.



FIGS. 9 TO 11. Camera lucida drawings of translocation complexes.

FIG. 9. The two complexes of four which were photographed in Fig. 3. In the drawing the bivalents are omitted. The arrows indicate the centres of the crosses. FIG. 10. Pachytene stage of an association of six chromosomes in the form of a double cross. FIG. 11. Double-cross association of six at diplotene.

The pachytene stage in a nucleus with two complexes of four and three pairs of chromosomes is photographed in Fig. 3. The centres of the cross-shaped complexes of four chromosomes are indicated by the arrows. The two complexes from this same cell are drawn in Fig. 9, one being represented by solid lines and the other by broken lines; for the sake of clearness the

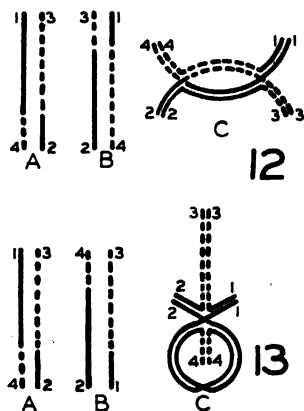
bivalents are omitted in the drawing. It will be observed that the association of homologous parts is close right up to the loci of interchange. This is true of all the different complexes of four observed at pachytene. The cross-shaped configuration is usually readily evident at the central part of the complex, but the long arms are so curved and twisted as to destroy the shape of the cross for the complex as a whole.

An important point in relation to sterility is the position of the breaks that led to the interchange. This can be determined from the relative lengths of the arms of the cross. In Fig. 9 it will be observed that in each complex two arms are about half as long as the other two. Actual measurements of several specimens of one complex give the proportions 1.0 : 1.3 : 2.1 : 2.1. Since the original chromosomes were of nearly equal length and both had median centromeres, the break in one chromosome must have occurred at a point about one-third the distance along one arm from the centromere, and in the other chromosome somewhat nearer the centromere. Examination of other translocations shows a considerable range in the position of the breaks. In the majority all four arms are nearly equal (Fig. 4); in such translocations both segments exchanged must have consisted of nearly complete arms of the original chromosomes and the breaks must have occurred very near the centromere. But in others one arm of the cross is very short and the other three long; in such cases one segment must have been very short and the other long; the break in one chromosome must have been near the end and in the other near the centre. In general it may be said that there is a strong tendency for breaks to occur near the median centromere but various other positions are represented.

The diakinesis stage in a nucleus with two complexes of four and three bivalents is photographed in Fig. 4. With the contraction of the arms the cross configuration becomes more symmetrical. Both complexes are beginning to loosen. It is important to note that the complex usually does not open out into a flat ring but changes more or less directly from the cross configuration to a figure of eight. This is in strong contrast to the behaviour in maize where, according to published accounts, apparently all complexes pass into the flat ring. The difference appears to be associated with a difference in the stage at which the opening out occurs. According to Anderson (1) the separation begins in maize in late pachytene or early diplotene, and at diakinesis only the ends are joined to form a typical ring. In wheat the separation occurs considerably later, and at diakinesis relatively few open rings are visible. Usually the complex appears to pass from a partly open cross directly to a figure of eight.

At metaphase the great majority of complexes are in the form of a horizontal figure of eight, which may readily be mistaken for two bivalents in contact. This is the position for alternate, disjunctional segregation. A small proportion form open flat rings. Some have broken at one point and form zigzag chains. Fig. 5 is a photograph of a cell showing a zigzag chain and a figure of eight at the right and three bivalents at the left.

Both the figure of eight and the zigzag chain must segregate in alternate fashion and therefore produce viable gametes. Of 100 complexes carefully studied for this purpose, 88 clearly had the disjunctional configuration. This agrees well with the data on sterility previously given.



FIGS. 12 AND 13. Associations of four due to two different translocations involving the same chromosomes.

FIG. 12. A, chromosomes resulting from first translocation which involved Ends 2 and 4; B, those resulting from the second translocation which involved Ends 1 and 3; C, the association in a plant heterozygous for both. FIG. 13. A, translocation involving Ends 2 and 4; B, involving Ends 1 and 4; C, the association in a plant heterozygous for both.

A matter that has apparently not been explored is the form that a complex would take in a plant that is heterozygous for two different translocations involving the same chromosomes. Of course if the points at which both chromosomes break are exactly the same in both translocations, no complex but only pairs will be formed in the F_1 . But if the points of interchange in either or both chromosomes are different in the two translocations, a single complex of four should appear in a plant that is heterozygous for both. This complex should, however, be very different from the simple cross of a single reciprocal translocation. Fig. 12 illustrates the situation when the two translocations involve the opposite ends of both chromosomes. In the first translocation (Fig. 12A), End 2 of Chromosome 1.2 and End 4 of 3.4 have been exchanged; in the second (B), Ends 1 and 3 have been exchanged. The form that the complex should take in a plant heterozygous for both is shown in Fig. 12C. Fig. 13 illustrates the situation when both translocations (A and B) involve the same end of one chromosome (End 4) but opposite ends of the other (Ends 1 and 2). In the cases illustrated in these figures, both segments exchanged in the first translocation are of the same length, and this length is the same as that of both segments exchanged in the second translocation. But any combination of lengths of these four segments may presumably occur, and any combination of ends. All these numerous variations should theoretically result in complexes of four that would be of different

and often complicated form. Since the chances of both breaks for both translocations occurring at identical points are very small, some such configuration in place of the simple cross or two pairs, is to be expected. In none of the material of *T. monococcum* have the same two chromosomes been involved in two different translocations. But in *T. durum* a case of the type illustrated in Fig. 12 has been produced.

IN COMPLEXES OF SIX

The form that a complex of six chromosomes takes during prophase is usually reported to be a star, as shown in Figs. 2 and 6. Actually this configuration occurs only when the two breaks in the chromosome that is involved in both translocations occur at the same place. If the piece transferred in the first translocation is from the opposite arm to that transferred in the second, the complex of six in the plant that is heterozygous for both will take the form of two crosses with one segment in common, as illustrated in Fig. 14. The double-cross configuration will also be produced if the segments from

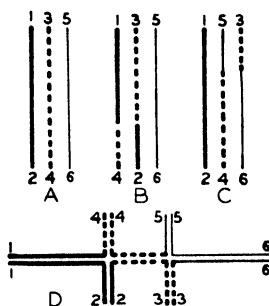


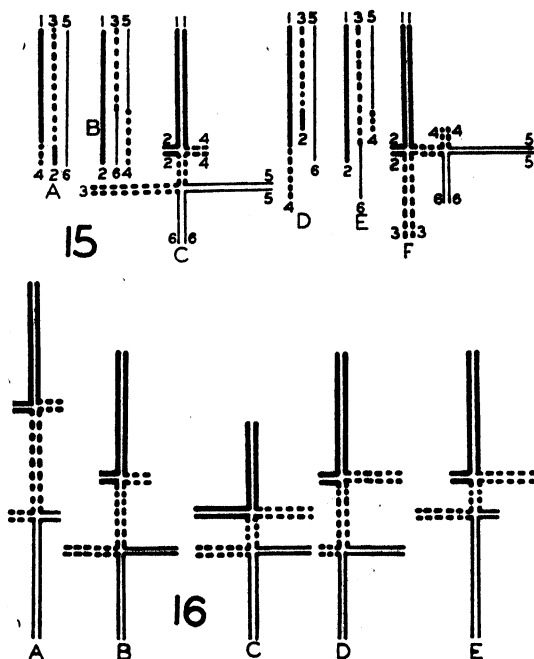
FIG. 14. A, the original chromosomes; B, after the first translocation; C, after the second; D, double-cross configuration in a plant heterozygous for both.

the common chromosome that are transferred in the two translocations are from the same arm but are of unequal length. This is demonstrated in Fig. 15. Unless there is a marked tendency for the different breaks to occur at the same place, the double cross should be much more frequent than the star. Of course transitions between the two configurations should occur when the pieces transferred from the same arm are unequal but not very markedly so.

An actual pachytene double-cross configuration is drawn in Fig. 10. All parts are represented in their proper positions and relative lengths, as drawn with the aid of a camera lucida. A diplotene stage is shown in Fig. 11. Naturally the double-cross configuration is usually distorted, particularly in the earlier, uncontracted stages, by the curving and twisting of the arms.

The form of the double cross varies greatly with the lengths of the segments that were translocated, as these determine the lengths of the different arms of the cross. This point is illustrated by the diagrams in Fig. 16, which show a few of the configurations produced from the same three original chromo-

somes by varying the lengths of the translocated segments. In each configuration the chromosome involved in both translocations is the one represented by the broken line, and the two segments translocated from it are from opposite ends. There are similar variations when segments of unequal length are transferred from the same arm of the common chromosome. Two of these are illustrated in Fig. 15.



FIGS. 15 AND 16. Some forms of double-cross configurations of six chromosomes, depending on the positions and relative lengths of the segments exchanged.

FIG. 15. The segments of the common chromosome are from the same end in both translocations. A, first translocation, short segments; B, second translocation, long segments; C, resulting complex; D, first translocation, short-long; E, second translocation, short-long; F, resulting complex. FIG. 16. The segments of the common chromosome (broken line) are from opposite ends in the two translocations. Various forms due to differences in the relative lengths of the segments.

A cell with a star-shaped complex of six and four pairs is photographed in Fig. 4. Usually the star shape of the central portion is readily evident, but the arms are so curved and intertangled as to destroy the star shape for the complex as a whole, particularly at earlier stages. The relative lengths of the arms vary with the length of the segments translocated but the two segments transferred from the common chromosome in the two translocations must be nearly equal or a double cross instead of a star will be produced.

Whatever form the complex of six may take during prophase, it is a zigzag ring at metaphase in the great majority of cases. Fig. 7 shows a cell with

such a ring and the four bivalents. Owing to the failure of a terminal association the ring is sometimes replaced by a zigzag chain as illustrated in Fig. 8.

Because of the zigzag form, whether of ring or chain, which is typically present at metaphase, it is obvious that segregation of complexes of six is normally alternate. This is true of the complexes in all the nine F_1 combinations that have been studied. Spores resulting from such segregation should be competent. Of 75 complexes carefully examined at metaphase 56 (74.6%) showed the complete disjunctional arrangement.

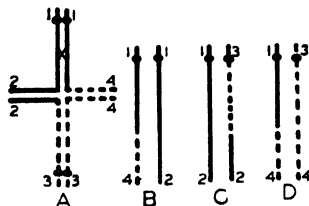


FIG. 17. Effect of crossover between the centromere and the point of interchange. A, prophase configuration with a crossover in Arm 1; B, chromosomes resulting from the crossover; C and D, chromosomes of the spores that would result from alternate segregation. Both have a deficiency and a duplication.

A considerable number of offspring (F_2) of plants that had complexes of six (F_1) were examined. In every pollen mother cell examined at a suitable stage in all these plants there were either seven pairs of chromosomes or a complex of six and four pairs. Therefore, if any gametes are formed through non-disjunctional segregation with more or fewer than the normal number of seven, or with the normal number but abnormal constitution, they are unable to produce viable offspring. Only two types of F_1 gametes function successfully; one has one of the two translocational arrangements that went into the cross and the other has the second. F_2 plants with seven pairs were fully fertile; those with complexes of six showed an average sterility of 21.1%, in close agreement with F_1 .

Discussion

The cytological studies show that the reason for the lack of sterility in wheat plants which are heterozygous for a translocation is that segregation is not random but directed. It is directed not merely by the movement of homologous centromeres to opposite poles, but also as a rule by the movement in opposite directions of adjacent chromosomes that have non-homologous centromeres. If such alternate segregation were invariable, only fully competent spores would be produced and fertility would be complete. But, in a small percentage of cells with associations of four and, in a somewhat larger percentage with associations of six something interferes with this normal procedure or in some other way causes sterility.

In order to explain why segregation is regularly disjunctional, it is only necessary to assume that the repulsion that causes segregation involves the

whole bodies of chromosomes and not merely the centromeres as is often postulated. If every chromosome repels those on either side of it, the alternate movement would result. And if the repulsion of the two homologous members of ordinary pairs involves more than the centromeres, it is to be expected that every chromosome in the ring would repel its neighbour on either side because adjacent portions of adjacent chromosomes are homologous, as shown by the fact that they are closely paired in prophase to form one arm of the cross.

On the other hand, in order to explain regular alternate segregation on the basis of centromere repulsion alone, it is necessary to assume that every centromere is influenced by one that is not homologous with it. Any centromere in the ring is homologous with that of the chromosome on one side only and yet it must be influenced by the one on the opposite side also, if alternate segregation depends on the centromeres. But there is no reason to think that any centromere can influence the direction of movement of any non-homologous centromere. Mendel's principle of independent assortment implies that there is no such influence. The normal occurrence of alternate segregation from translocation complexes therefore shows that the forces that cause chromosome segregation involve whole chromosomes (or at least their ends) and not merely the centromeres. Presumably this is true of chromosome pairs as well as complexes.

If the alternate segregation in wheat, followed by a high degree of fertility, is understandable on these general conceptions, there is immediately encountered the difficulty that in *Zea* there is 50% sterility and, according to McClintock (9), adjacent as well as alternate segregation. The situation in *Pisum* is similar, according to Håkansson (7). It is not to be expected that the basic mechanism of chromosome movement would be so different in different genera that one would show only alternate and another random segregation. It is more reasonable that the segregation is basically alternate (disjunctional) but that in some cases special conditions interfere and give the appearance of random segregation.

This opinion is supported by several considerations:

(1) In maize the segregation is not really random, for, if it were, the sterility would be 66.6 instead of 50% in cases of rings of four and 90 instead of 70% in cases of rings of six. Of course it may be maintained that randomness is restricted only to the extent that homologous centromeres must go to opposite poles. But once the complexes have opened out into rings, there is no apparent reason why homologous centromeres must necessarily go to opposite poles. Moreover, McClintock (9) showed that they may actually go to the same pole.

(2) The translocations that have been studied in maize were discovered because they cause semisterility. Search is made for them by examining semisterile plants. Consequently, only those with non-alternate segregation are discovered. It is possible that there may be others with alternate segregation, as in wheat, that have not been found. On the other hand the trans-

locations in wheat were discovered through a systematic cytological examination of the offspring of radiated plants (the only way they could be discovered) and their fertility was proved later. It would be of interest to know whether a systematic cytological examination of the offspring of radiated maize would reveal the occurrence of fully fertile translocation heterozygotes in that genus. In this connection it is important that Clarke and Anderson (4) mention the occurrence in maize of a few translocations involving sterility from 19 to 50%, and Burnham (3) reports a case with 25%.

(3) In certain other genera disjunctional segregation is the rule or is interfered with only enough to produce less than 50% sterility. The zigzag segregation of the large rings of *Oenothera* is well known. Gairdner and Darlington (6) found 30% non-disjunction in a case in *Campanula*. Sax and Anderson (11) reported that different translocations in *Tradescantia* exhibit different amounts of adjacent segregation. According to Bergner, Satina, and Blakeslee (2) most of the translocation heterozygotes in *Datura* are fully fertile. In different translocations in the same and in different genera, alternate segregation seems to be prevented to different degrees. Even in *Triticum* disjunctional segregation is not invariable and fertility is not quite complete.

Factors that cause sterility by interfering with alternate segregation or in some other way, should be revealed by comparing, with respect to chromosome structure and behaviour, those forms like maize and peas that show semisterility with those like wheat that do not. Such a comparison brings out the following points:

(1) In the bivalents of maize there are numerous interstitial chiasmata, and the same is true in *Pisum*. In wheat, on the other hand, both in bivalents and complexes, the chiasmata are usually terminal, although a few interstitial ones may be found. The situation is the same in *Oenothera*, *Datura*, and *Campanula*, the other genera in which examples of alternate segregation or low sterility have been reported. As Darlington (5) pointed out, the occurrence of interstitial chiasmata would interfere with the opening out and orientation of the complex on the metaphase plate. On the other hand the data of Sax and Anderson (11) from *Tradescantia* fail to reveal a correlation between the number of interstitial chiasmata and the amount of non-disjunctional segregation. Their cases may, however, have been complicated by another factor to be considered later.

(2) In spite of the larger number of interstitial chiasmata in maize, the cross appears to open out earlier than in wheat. According to Anderson (1) the opening out begins in maize in late pachytene or early diplotene and by diakinesis only the ends of the chromosomes are in contact, so that open flat rings are characteristic of diakinesis. Perhaps the examples on which these observations were made were ones without the interstitial chiasmata which would be expected to prevent the opening out. In wheat, although there are fewer interstitial chiasmata, relatively few flat rings are to be seen at diakinesis. Usually the complex seems to change directly from a cross to a

figure of eight without passing through an open-ring stage. If the repulsive forces operate strongly at an early stage and produce a flat open ring before orientation has begun, as appears from published descriptions to be the rule in maize, there might be little tendency for adjacent chromosomes to move in opposite directions. But if the repulsion of homologous parts takes place only later, after orientation has begun, the figure of eight would naturally be formed as in wheat, and adjacent chromosomes must go in opposite directions. It is clear, however, that this cannot be a general explanation for all types, because in *Oenothera* the complexes regularly pass into very large, widely open rings and thence into the zigzag condition.

(3) In most of the chromosomes of maize the centromeres are not median; the same is true in *Pisum*. In *Triticum monococcum*, on the other hand, all seven have median centromeres as have those of *Datura* (10), and most of those of *Campanula* and *Oenothera*. When the centromere is in the middle of the chromosome, the distance between it and the point of interchange is likely to be less than when it is near the end. Consequently, crossovers are likely to be fewer between a median centromere and the point of interchange. Fig. 17 shows that chiasmata in this region must have difficulty in terminalizing, and, when they do, all the gametes resulting from alternate segregation will be inviable. The position of the centromere near the end of the chromosome therefore tends to cause sterility through the occurrence of crossing over between the centromere and the locus of interchange.

The same effect would be produced even though the centromere were median, if at least one of the segments translocated were short. A short segment necessarily means a considerable distance between centromere and point of interchange, and hence more frequent crossovers in that region. But there is no reason to expect that the length of the segments exchanged should be greater in wheat than in maize or peas. As a matter of fact in most of the translocations studied in wheat the segment consisted of nearly a whole arm. But in some the segment was very short, and yet the sterility was low.

If the chromosome as a whole is short, the distance between centromere and locus of interchange is likely to be short, and hence the frequency of crossovers in that region low. This may be a factor in *Oenothera* and *Datura* which have small chromosomes, in contrast with *Zea*, *Pisum*, and *Tradescantia* which have long ones. But *Triticum* also has long chromosomes.

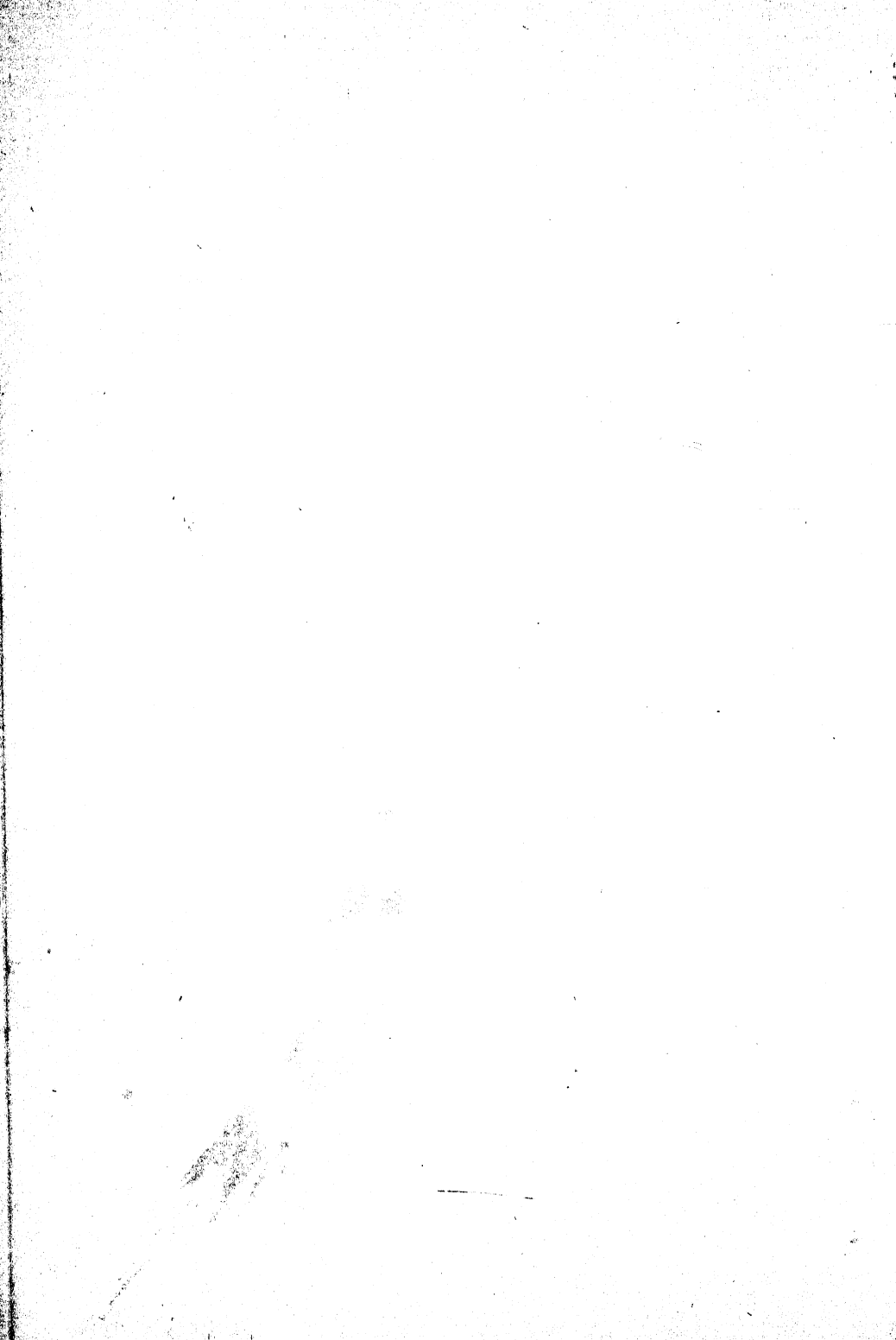
Thus some of the factors that may be expected to cause sterility in translocation heterozygotes are (1) slow or incomplete terminalization of chiasmata, (2) early separation of homologous parts, (3) non-median position of the centromere, (4) shortness of segments exchanged, (5) great length of chromosomes. Since these factors are variable from species to species, and some of them from case to case within a species, it is to be expected that the degree of sterility will vary. The 50% frequently reported has no special significance. Wheat has only isobrachial chromosomes; its chiasmata are mostly terminal; in most of the translocations studied the segments exchanged are long; and

the separation of homologous parts is delayed so that usually the open ring is omitted. Hence sterility in wheat is low. On the other hand maize has several heterobrachial chromosomes in which many interstitial chiasmata occur and open rings are regularly formed; hence numerous cases with high sterility are observed.

In complexes of six or more chromosomes additional factors come into play. This is evident from the fact that plants with one complex of six are consistently more sterile in wheat than those with two complexes of four, although in both types only two interchanges are involved. Presumably the greater unwieldiness of the larger complexes makes regular orientation more difficult. In addition, it should be remembered that the configuration will usually be a double cross and not a star, unless there is a marked tendency for different breaks to occur at the same locus. In the double cross each of two chromosomes must pair with three others, and this may involve difficulties. Moreover, crossovers in the segment that connects the two crosses will interfere with orientation and will always produce incompetent combinations of chromosomes.

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FURTHER STUDIES OF OSMOTIC AND PERMEABILITY RELATIONS IN PARASITISM¹

By F. S. THATCHER²

Abstract

Changes in host cell permeability induced by fungus parasites selected from several distinctive types of disease relationship were studied by plasmolytic methods.

Puccinia graminis Tritic race 21 causes an increase in permeability to cells of the susceptible wheat varieties, Mindum and Little Club.

Resistance of Mindum wheat to race 36 is associated with a local decrease of host cell permeability.

Narcotization of Mindum wheat increases permeability and renders this variety more susceptible to race 36 of *Puccinia graminis Tritic*. This information, together with a hypothesis, already expounded, explaining mechanisms involved in food uptake by rusts, was used in the formulation of a theory illustrating a basic component of the factors responsible for rust resistance. This theory seems to interrelate the two main contrasting theories hitherto propounded.

Permeability increase is also demonstrated as an effect of tissue invasion by *Botrytis cinerea*, *Sclerotinia Sclerotiorum*, and *Phytophthora infestans* on their respective susceptible hosts. This fact is allied with other information to explain the characteristic symptoms associated with the diseases caused by these fungi, and to propose an accessory role of permeability increase in the parasitism of these organisms.

The probable cause of wilting induced by hadromycotic fungi is discussed, and the role of a permeability increase demonstrated for leaf cells of tomato subjected to the action of a filtrate of a culture of *Fusarium Lycopersici* is discussed in this connection.

A decrease found in the permeability of tissues of swede "root" near the margin of a necrotic lesion caused by *Phoma lingam* was interpreted as being a change in accord with Brown's suggestion that a dry rot is determined by the ability of the host plant to restrict the amount of water reaching the parasite and so arrest the progress of its enzymic activity at some intermediate stage.

For a full understanding of parasitism, a knowledge of the processes governing the intake of water and food substances by parasites from the tissues of their hosts is essential. Nevertheless, a comprehension of this aspect of the nutrition of phytopathogens is decidedly incomplete.

The greater part of the information on parasitic relationships has been derived from studies of entire plants or organs, and from histological examination of fixed preparations. It seems to the author that such subjects impose

¹ Manuscript received February 2, 1942.

Contribution from the Faculty of Agriculture, McGill University, Macdonald College, Que. Based on part of a thesis submitted to McGill University in 1939 in partial fulfilment of the requirements for the degree of Doctor of Philosophy. Journal Series No. 168.

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a decided limitation to the possible scope of inquiry, and that the investigational horizon could be considerably broadened if the functional unit of the plant—the living protoplast—were given more consideration as an investigational unit.

Attempts have been made in this and in an earlier paper (34) to gain further insight into the mechanisms of parasitic nutrition by a study of vital cellular phenomena.

The aggressive action of a parasite is essentially an expression of a particular mode of nutrient acquirement. It seems reasonable, therefore, to expect that phenomena that may condition the ability of the parasite to assimilate its required foods and water may well be influential in determining the outcome of the antibiotic struggle which "parasitism" connotes; that is, they would contribute to the determination of resistance or susceptibility.

Accordingly, investigations of osmotic and permeability relationships in various host-parasite associations have been continued with an ultimate purpose of obtaining information that might serve as a basis for further research into the factors governing disease resistance.

An investigation of this type should be particularly pertinent in its application to the rusts since the rust fungi obtain their food materials from living cells only. It is generally conceded that the rusts do not modify the cell walls of their hosts; neither is there evidence to suggest that the substance of the host protoplasm is utilized. Consequently, the source of food would seem to be the vacuolar contents. These are held within a semipermeable membrane. Since the active rust thallus is essentially intercellular it is to be inferred that survival of the rust fungi in their parasitic environment is dependent upon their ability to overcome, at least partially, the semipermeability of the host cell plasma membrane. The possibility that the presence of haustoria may modify the intercellular nature of the fungus is not considered likely, as will be discussed later.

The rather scanty literature attaching significance to osmotic pressure and permeability in discussions of parasitism already has been reviewed by the author (34). Results, reported at that time, of a study of osmotic and permeability relationships between a number of fungi and their hosts indicated that (1) in each instance the osmotic pressure of the fungus was greater than that of its host, and (2) whenever a susceptible host was attacked each of the parasites investigated caused an increase in permeability to both water and solutes of the plasma membrane of contiguous host cells.

From these data a hypothesis was elaborated to explain the mechanism of transfer of food and water from the cells of a host plant to those of the parasite as manifest in the rusts investigated (*Uromyces Fabae* (Pers.) de Bary and *U. caryophyllinus* (Schr.) Wint.), and to indicate also that osmotic pressure and permeability relationships play a fundamental role in the parasitism of more drastically destructive fungi such as *Botrytis cinerea* Pers. and *Sclerotinia Sclerotiorum* (Lib.) Mass. This same hypothesis permits explana-

tion of the extended survival of parasitized cells such as is general among the rusts.

For the argumentative development of this hypothesis the reader is referred to the original paper, but, briefly, it is suggested that the higher osmotic pressure of the fungus enables it to obtain water from neighbouring host cells, and that an increase in permeability renders available to the fungus those cell solutes to which the plasma membrane no longer demonstrates the property of semipermeability.

The rusts and the soft rot fungi represent two contrasting types of parasites. The former is dependent upon the continued survival of its host, the latter rapidly exerts a lethal virulence. Data on these two groups have been amplified, and, in order to make this investigation more comprehensive, the present study has been extended to diseases caused by organisms typifying intermediary degrees of specialization in their mode of parasitism and associated with diverse types of symptom expression.

Selections were made from the following conventional groups of phytopathogens: (1) obligate parasites—(a) rusts, (b) powdery mildews; (2) parasites that by some diffusive influence kill the cells of their hosts in zones beyond the region occupied by their mycelium—(a) soft rot fungi, (b) dry rot fungi ("hard rot" in the sense of Brown (4)); (3) parasites whose hyphae extend beyond any visible sign of host cell necrosis; and (4) those parasites associated with the diseases known as hadromycoses whose principal symptom is a foliage wilt.

The hadromycoses were included in this study, not only because they are a specific and widespread group of diseases but because permeability change, though suggested as a possible factor in the cause of wilting, has never been demonstrated in this connection.

Materials and Methods

The species chosen to represent the types of pathogens described above are listed below:—

(1) *Obligate parasites.* *Puccinia graminis Triticis* Erikss. and Henn., causing stem rust of wheat was chosen to augment the data relative to rusts already reported. Two physiological races were used: race 21, which on Mindum wheat provides a type IV reaction, and race 36 to which Mindum may give type 0 or type I reactions. Mindum wheat is thus highly susceptible and highly resistant, respectively, to the two rust races, the reactions being estimated in accordance with Stakman and Levine's (31) directions. The powdery mildew used was *Erysiphe Polygoni* DC., growing upon the leaves of swede turnip (*Brassica rutabaga* L.).

(2) *Fungi causing contrasting types of disease.* *Botrytis cinerea* Pers. and *Sclerotinia Sclerotiorum* (Lib.) Mass. induce typical "soft rot" symptoms when growing upon mature celery petioles (*Apium graveolens* L.), while *Phoma lingam* (Tode) Desm. caused a typical "dry rot" of swedes under the conditions

of the experiments reported below. This latter disease corresponds to a "hard rot" in the sense of Brown (4).

(3) *Phytophthora infestans* (Mont.) de Bary was used as an example of the third group of parasites mentioned above, since, 30 hr. after inoculation of potato petioles (*Solanum tuberosum* L.), intercellular distributive hyphae were found to extend 1300 μ beyond the slight necrosis around the incision made at the point of inoculation.

(4) *Fusarium Lycopersici* Sacc. (*Fusarium bulbigenum* var. *Lycopersici* (Bruishi) Wr.) was selected from among the many wilt pathogens chiefly because of the extensive studies with this organism already reported in connection with the wilt of tomatoes (*Lycopersicon esculentum* Mill.) which is a typical disease of its kind.

The treatments accorded the various host subjects prior to examination and the actual tissues used are indicated below.

1. Wheat

Mindum wheat was grown in 4½-in. pots, three plants to a pot, and was inoculated with urediniospores applied with a small scalpel after the method of Stakman as described by Lehmann *et al.* (19). The inoculum of race 36 was applied very heavily in order to provide a dense distribution of the "flecks" characteristic of a resistant reaction, so that the considerable difficulty of locating cells in the immediate vicinity of rust hyphae would be minimized. Extra light was supplied to the plants by a battery of 100-watt bulbs which were used from 5 p.m. to midnight throughout the life of the plants. The temperature of the greenhouse was maintained close to 65° F. Sections were cut from wheat inoculated with either race about 12 to 15 days after inoculation. Leaves compared were from the same nodes of respective plants and from plants of the same age, unless it was possible to obtain diseased and healthy tissue from mature parts of the same leaf. The average osmotic and permeability values, however, showed practically no difference from plant to plant of the same series, though differences between plants of different ages were apparent. Leaves from several plants were used until data on several hundred cells had been accumulated, the cells studied being of mesophyll tissue. The sections were cut parallel to the veins. The irregular shape of the mesophyll cells prevented determination of absolute permeability.

2. Swedes

Mature swede "roots" taken from storage were the subject for the tests with *Phoma*. These had been coated prior to storage with a thin layer of paraffin, and attention was attracted by the sunken, dry rot lesions often below the unbroken surface of the paraffin. The pathogen responsible for these lesions was determined as *Phoma lingam* (Tode) Desm. The disease seemed particularly worthy of investigation because of the difficulty of explaining the complete absence of free water among the killed tissues even though evaporation was reduced to a minimum by the intact paraffin layer. The

lesions often developed into locally ingressive necrotic "pockets" which were sharply demarked from the healthy tissues of the root. Cavities formed by the disintegration and collapse of cell walls were evident in these "pockets". Sections were cut in such a way as to include part of the collapsed tissue as well as normal tissue beyond this region. A few hyphae were found to extend short distances into this apparently healthy tissue (xylem storage parenchyma). The necrotic tissue and the cavities described above were filled with densely intertangled mycelium. Measurements of solute permeability were made from cells situated at from two to five cell diameters away from the edge of the collapsed tissue. Measurements of healthy cells were made from the same sections at some distance from the diseased zone, and also from sections of healthy tissue from the same root, the test cells in this instance being in the same position relative to the normal periderm as were the cells bordering the lesion.

For the tests with *Erysiphe*, naturally infected leaves of swedes growing in sand culture were used. The relative permeability to water of infected and non-infected epidermal cells was estimated from strips of epidermal tissue torn from the leaves, and, later, from epidermal tissues freed from underlying leaf tissues by careful scraping as described by Allen and Goddard (1). The mildew haustoria penetrate only into the epidermal layer, but measurements were also made in subepidermal tissue in order to attempt to relate a possible permeability change with the increased respiration found by Allen and Goddard (1) to be induced in these tissues as a result of epidermal infection. Accordingly, relative permeability of palisade cells to water was estimated from sections cut from areas of extensive mildew infection and from uninfected regions of the same leaves.

3. Celery

Petioles of mature celery plants taken directly from cold storage were washed repeatedly in sterile water and inoculated with *Botrytis cinerea* and *Sclerotinia Sclerotiorum* originally isolated from decaying celery. The petioles were kept at room temperature in covered gas jars partly lined with moist filter paper. The lower ends of the petioles rested on wire platforms in order to prevent contact with any fluid accumulating at the bottom of the vessels. Sections were cut at different intervals from the developing lesions about 7 to 10 days after inoculation. Relative permeability to water was estimated from the cortical parenchyma cells.

4. Potato

Petioles of the larger leaves of Green Mountain potato plants growing in a greenhouse at 65° F. were inoculated, in situ, with hyphae and spores of *Phytophthora infestans* grown in pure culture on potato tuber. The inoculum was introduced aseptically into a small incision made with a spear-head dissecting needle. As soon as extensive necrosis had become evident, permeability to water and to dextrose was estimated from cylindrical, chlorenchymatous, inner cortical parenchyma cells appearing in sections cut at the

border of the necrotic zone, and from similar cells of healthy regions of the same petioles.

5. Tomato

Tomato plants grown in soil inoculated with *Fusarium Lycopersici* after the method of Clayton (6) did not develop typical wilt in the time available for this phase of the present work. Accordingly, excised stems were placed in a filtrate obtained from four-week old cultures of the fungus in Haymaker's (14) modification of Richard's medium.

In order to preclude any possibility of mechanical obstruction by germinating spores, sterile filtrates were obtained by the use of a Seitz filter. Wilting occurred in about 48 hr. The test leaves were not only completely flaccid, but also showed some degree of necrotic withering at the leaf tip or margins. Measurements were made from check plants treated identically except that they were placed in water. Determination of water permeability was made using the relatively large, cylindrical, sparsely chlorophyllose cells that lie near and parallel to the conducting elements of the veins, as well as the palisade parenchyma, both from large leaves of well grown plants. The ideal regular cylindrical shape of the cells first measured made calculation of absolute permeability possible, but the palisade cells were too irregular to warrant estimates other than of relative permeability.

Osmotic Pressure Determinations

As in earlier studies, the osmotic pressures of the respective hosts and parasites were compared by means of the plasmolytic method (34), which is dependent upon some means of estimating solutions isotonic with the test cells.

The relative superiority of the osmotic pressures of all parasites investigated as compared with the values for their respective host cells is indicated in Table I, and is in conformity with the measurements to which reference has already been made. Values already reported are included in this table for the sake of comparison.

Permeability Determinations

Permeability tests already made by the writer, using the plasmolytic method, have shown that in specific instances parasitism induces permeability change to various test materials (water, urea, thiourea, and dextrose). Permeability to a number of test solutes, therefore, was not determined for each pathogen since the solutes used are of similar polarity relative to water, and, hence, it may be argued that an increase in permeability to urea, for example, indicates an increase in permeability to water, or vice versa. Accordingly, in order to reduce time consuming duplication, urea was more generally used as a test substance for permeability change because it permits a fairly slow deplasmolysis with little injury to protoplasts, and also because the deplasmolysis times are sufficiently long to facilitate accurate measurement.

TABLE I
OSMOTIC PRESSURE OF PARASITE AND HOST

Parasite		Host	
Fungus	Average O.P., atmospheres	Host plant (healthy tissue)	Average O.P., atmospheres
<i>Uromyces fabae</i>		<i>Pisum sativum</i>	
Germ tubes	44.25	Leaf	9.15
Hauatoria	21.90	Petiole	10.10
<i>U. caryophyllinus</i>		<i>Dianthus</i>	
Hauatoria	18.6	Leaf base	11.2
<i>Puccinia graminis</i>		Mindum wheat	
Hauatoria (race 21)	18.9	Leaf	9.4
<i>Erysiphe Polygoni</i>		<i>Brassica</i>	
Hyphae	18.0	Leaf	10.6
<i>Phytophthora infestans</i>		<i>Solanum</i>	
Hyphae (aerial)	17.4	Tuber	10.6
Hyphae (intercellular)	15.5	Petiole	8.9
Sporangia	18.1		
<i>Botrytis cinerea</i>		<i>Apium graveolens</i>	
Hyphae	29.8	Petiole	8.3
<i>Sclerotinia Sclerotiorum</i>		<i>Apium graveolens</i>	
Hyphae	23.5	Petiole	13.4 (9.4 - 17.4)
<i>Phoma lingam</i>		<i>Brassica</i>	
Hyphae	41.3	Root	11.3

Permeability Changes Induced by Puccinia graminis Tritici in Susceptible and Resistant Wheat Varieties

An increase in permeability to urea is induced in wheat varieties when attacked by a particular physiological race of stem rust to which the varieties are susceptible. The cells of Mindum wheat which are in close proximity to an established thallus of race 21 show an approximately twofold increase in urea permeability, Mindum being highly susceptible to this race. This relation is expressed graphically for two series of test plants in Fig. 1, by plotting the percentage of cells of diseased and healthy tissues that had deplasmolysed in a hypertonic solution after different periods of immersion in that solution. A similar change is found when the susceptible variety Little Club is attacked by race 36. The increased permeability of infected tissue of this variety is expressed in a similar manner in Fig. 2. However, a decidedly different change is found when a rust develops in an uncongenial host variety. Mindum wheat is highly resistant (0 reaction) to *Puccinia graminis Tritici*, race 36, and instead of an increase in permeability being found in infected tissue as was the case with race 21, an extreme decrease was noted in each test with three series of plants. Permeability of cells in the

immediate vicinity of race 36 hyphae was so greatly decreased that very few cells would deplasmolyse at all. Fig. 3 indicates the progress of deplasmolysis, and the curves show that those cells that did completely deplasmolyse did so in a relatively short time, which suggests that they were either those least modified by the presence of the rust, or may even have been cells beyond the range of emphatic influence of the rust hyphae, and included in the measurements by error. After being plasmolysed for three to four hours, practically all the host cells near the fungus were dead, disintegration of the membrane occurring suddenly. The plasmolysed protoplasts appeared quite normal prior to their collapse, but in order to be certain that no unusual artifact was being observed, representative sections were transferred to hypotonic solutions, where in due time they deplasmolysed quite normally, thereby demonstrating that their failure to deplasmolyse in the hypertonic solution was a true indication of decreased permeability to the solute in question.

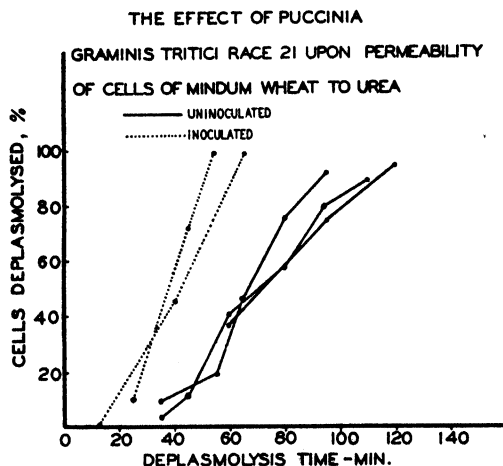


FIG. 1. Increase in permeability caused by *Puccinia graminis Tritici* race 21 on mesophyll cells of *Mindum* wheat.

In order to dispel all possible doubt that the small lesions examined may have been caused by some agency other than by infection with race 36, pieces of representative leaves were cleared and stained by Smith's (29) whole leaf method. The extent of the mycelium was easily determined as is evident from the photomicrograph of one such preparation shown in Fig. 8. Further proof is provided by Fig. 9 which shows the development of a minute sorus in the centre of a "fleck".

The Effect of Narcotization on Permeability of Cells of Mindum Wheat and upon Degree of Infection by Puccinia graminis Tritici, Race 36

Lepeschkin (20) has shown that concentrations of chloroform above 0.1% cause marked increase in permeability of cells of a number of plants. Similar effects of narcotics are mentioned by Stiles (32), and by other authors whose

THE EFFECT OF PUCCINIA GRAMINIS TRITICI
FORM 36 ON PERMEABILITY OF MESOPHYLL CELLS
OF LITTLE CLUB WHEAT TO UREA

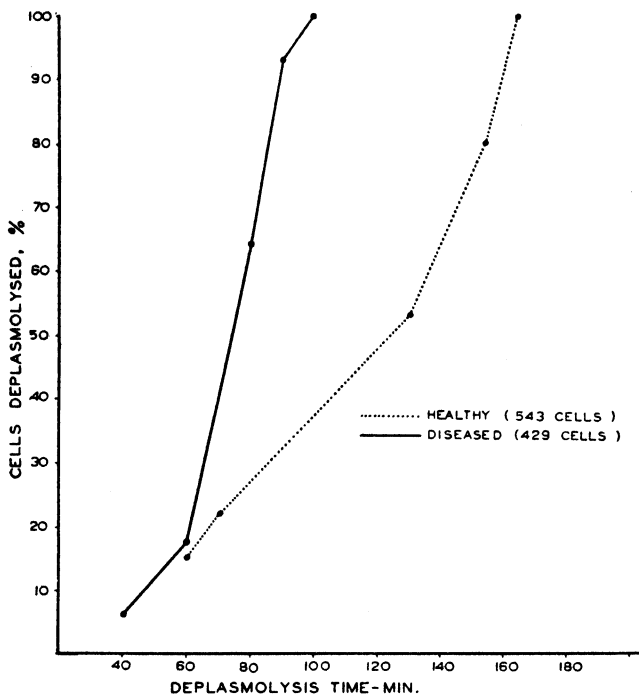


FIG. 2. Permeability increase caused by race 36 on Little Club wheat.

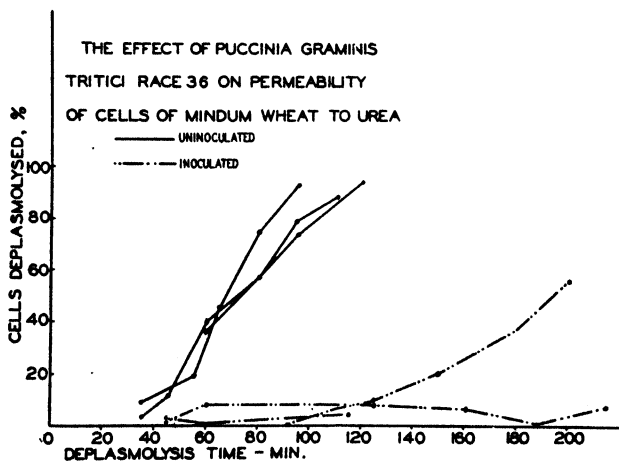


FIG. 3. Permeability decrease caused by race 36 on Mindum wheat.

work is reviewed by Winterstein (38). Stakman (30), and, more recently, Gassner and Hassebrauk (13) have shown that treatment of resistant varieties of cereals with chloroform after inoculation tends to increase the degree of host reaction to a particular rust race. Gassner and Hassebrauk (13) suggest that this latter change is due to a stimulated photosynthetic activity and an increased nitrogen content. However, in the light of the results already at hand at the time of publication of Gassner and Hassebrauk's work, it seemed desirable to determine whether change in rust reaction due to narcotization might be correlated with permeability change. Accordingly, Gassner and Hassebrauk's method of narcotization was adopted and measurements of urea permeability were made upon cells of Mindum wheat that had been narcotized, and upon cells of wheat that had been inoculated with race 36 (normal reaction = 0) and then narcotized two days later. Test plants were enclosed under large bell jars that had an opening for a stopper at the top. The bell jars rested upon a sheet of glass, the lower edge of each bell jar having been smeared liberally with vaseline in order to make an air-tight joint. A small pad of cotton wool was suspended from the upper stopper, and after placing the test plants under the bell jar, chloroform was applied to this cotton wool from a pipette at the rate of 0.1 cc. per five litres of enclosed air space. The stopper was then made air-tight with vaseline. Plants were kept in this atmosphere for two days, and permeability measurements were made within a few hours after their removal.

This narcotization treatment was found to increase the host reaction from a normal value of 0, to -1, to 1. The increased susceptibility conferred by

TABLE II

THE EFFECT OF CHLOROFORM VAPOUR ON PERMEABILITY OF CELLS OF
MINDUM WHEAT AND ON DEGREE OF INFECTION BY
Puccinia graminis Triticæ, RACE 36

Host treatment	Deplasmolysis time (min.)	Degree of infection
Normal wheat (untreated)		
Series 1	67	—
Series 2	71	—
Series 3	72	—
Narcotized	50	—
Infected with race 36 (not narcotized)		
Series 1	>200	0
Series 2	193	0
Series 3	>200	0
Infected with race 36 (and narcotized)		
Series 1	96	-1 to 1
Series 2	88	-1 to 1
Series 3	115	-1 to 1

this treatment is clearly evident in the photographs of untreated and narcotized rusted leaves of the same age which had been inoculated at the same time with inoculum from a common source (Fig. 10).

Results of permeability measurements of cells in the immediate vicinity of rust thalli in narcotized and in untreated plants are presented in Table II. As has already been indicated, urea permeability is extremely decreased by the activity of race 36 on Mindum wheat. The narcotization treatment, however, very much reduces the extent of this decrease, though the influence of race 36 is still apparent since the permeability of diseased cells of narcotized, infected wheat is still less than that of normal cells. Fig. 4 illustrates the action of the narcotic alone, the tests for which were made on the narcotized, uninfected plants within a few hours after removal from the chloroform. The treatment clearly induces an increase in permeability, though to a less extent than does inoculation with race 21 to which Mindum is highly susceptible.

The results of the wheat rust studies are collectively summarized in Figs. 4 and 5. Briefly, they are as follows: race 21, to which Mindum is susceptible, induces a marked increase in permeability; race 36, which induces a normal reaction of 0, causes an extreme decrease on the same host. Narcotization of plants infected by race 36 increases susceptibility and diminishes the extent of permeability decrease induced by this race alone. The narcotic alone increases permeability.

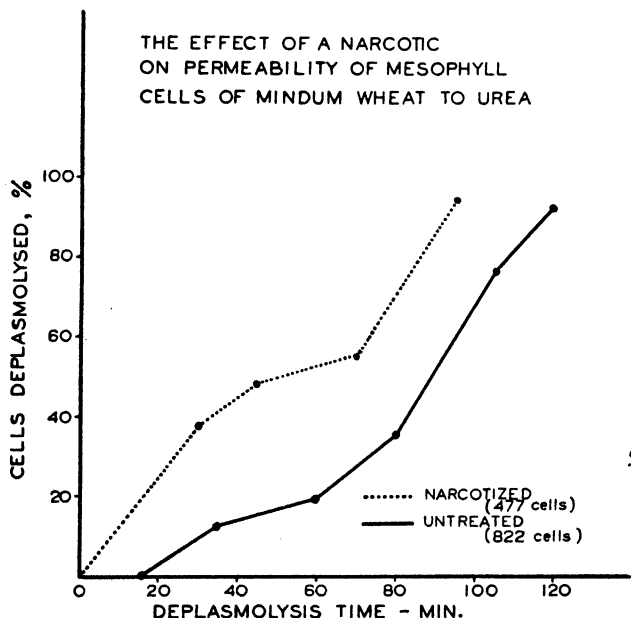


FIG. 4. Permeability increase caused by treatment of *Mindum* wheat with chloroform.

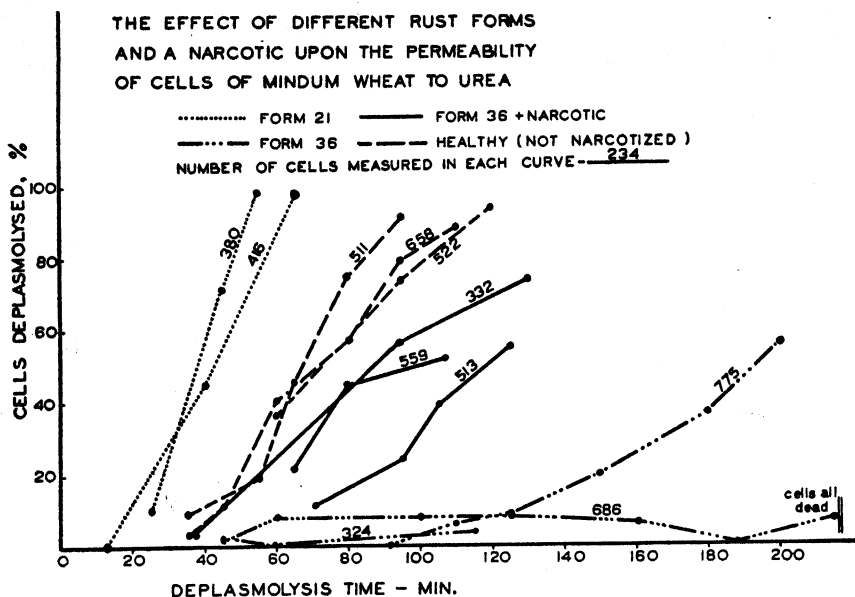


FIG. 5. A composite graph illustrating the comparative effects of races 21 and 36 to which Mindum wheat is respectively susceptible and resistant, together with the effect of a narcotic and the combined effect of a narcotic and race 36. All ordinates are expressed as the percentage of cells, in populations totalling about 100, having deplasmolysed after immersion for the stated times intervals in 1.5 M urea.

Permeability Changes Induced by *Erysiphe Polygoni*

The effect of *Erysiphe Polygoni* on the water permeability of palisade cells underlying an infected epidermis of leaves of *Brassica rutabaga* is shown in Table III. These values are expressed as frequency curves in Fig. 6, the curves being prepared by plotting the total numbers of cells that deplasmolysed during 25-sec. time intervals. (Total populations are the same for each curve.) It will be seen that practically all cells from infected regions show a more rapid deplasmolysis rate (greater permeability). One group of the healthy cells, whose presence renders the curve asymmetrical, constitutes an exception to this generality. It is possible that this asymmetrical region indicates experimental error perhaps induced by the lack of homogeneity among osmotic values of individual cells which may be found even within a single tissue.

Permeability studies of infected epidermal tissue would be of value at this juncture, but available time has not permitted the measurement of a sufficient number of cells to warrant presentation of figures.

Permeability Changes Induced by the Soft Rot Fungi, *Botrytis cinerea* and *Sclerotinia Sclerotiorum*

The experiments reported under this heading are practically a duplication of the permeability tests already described in connection with *Botrytis* and

TABLE III

THE EFFECT OF *Erysiphe Polygoni* ON WATER PERMEABILITY OF PALISADE CELLS OF SWEDE
(*Brassica rutabaga*) LEAF—PLASMOLYTIC METHOD

Palisade below uninfected epidermis		Palisade below infected epidermis	
No. of cells observed per section	Deplasmolysis time (sec.)	No. of cells observed per section	Deplasmolysis time (sec.)
4	185	4	90
12	155	7	125
2	165	4	100
6	110	5	150
4	160	10	70
2	100	5	105
12	165	7	85
12	165	3	130
10	120	3	90
6	200	4	80
2	220	3	115
10	120	3	90
7	160	10	105
4	155	10	150
18	140	2	110
7	160	15	110
6	160	10	95
8	155	5	90
		12	105
		5	90
		5	90
Total	132	132	
Average	147		103

Sclerotinia (34). The results are essentially the same but are reported to permit ready comparison of permeability changes induced by the representatives of the various groups of pathogens outlined at the beginning of this paper.

Examination of celery tissues attacked by each of the parasites *Botrytis cinerea* and *Sclerotinia Sclerotiorum* reveals that each fungus causes a four-fold increase in permeability to water of those cells just beyond the discoloured and largely necrotic zone which extends from the point of inoculation. These data are presented in Table IV, and are expressed as frequency curves in Fig. 7. The ordinates for the curves are obtained by indicating the number of cells that attain deplasmolysis during consecutive time intervals of 50 sec. Each curve is based upon the deplasmolysis times of 100 cells, individually measured. The practically identical nature of the curves for each fungus probably indicates that their mode of action is essentially the same, as are the earlier macroscopic symptoms of the disease caused by each on this particular host.

In order to compare the extent of permeability changes with the extent of enzymic activity in celery attacked by *Botrytis*, pieces of tissue were cut from infected petioles seven days after inoculation, at progressive distances from

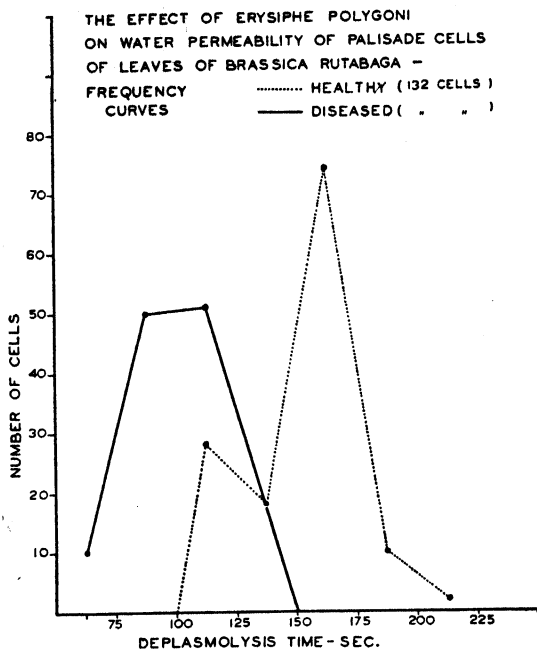


FIG. 6. The increase in permeability to water of palisade cells of *Brassica rutabaga* underlying an epidermis infected by *Erysiphe Polygoni*. Plasmolysed in 2 O calcium chloride; deplasmolysed in 9/10 O calcium chloride. O = the osmotic value of the cell sap of the cells under test.

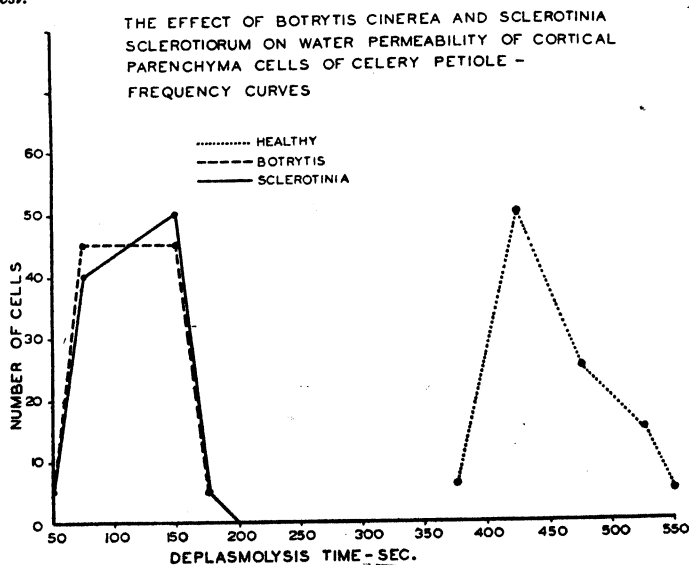


FIG. 7. The increase in permeability to water of cells of celery petiole within one inch of necrotic zones caused by *Botrytis* and *Sclerotinia*. Cells plasmolysed in 2 O; deplasmolysed in $\frac{9}{10}$ O calcium chloride.

the point of inoculation, and subjected to the Ruthenium red test for pectinase activity. Ruthenium red was used in accordance with the technique described by Rawlins (25). Pectin hydrolysis could not be determined in tissues more than two cell diameters away from obviously disintegrating cells in a trans-

TABLE IV

THE EFFECT OF *Botrytis* AND *Sclerotinia* ON WATER PERMEABILITY OF CORTICAL PARENCHYMA OF CELERY PETIOLE

Average deplasmolysis time of cells in each section of tissue (sec.)					
Healthy tissue		Tissue infected with <i>Botrytis</i> *		Tissue infected with <i>Sclerotinia</i> *	
Series 1	420	Series 1	90	Series 1	60
	570		90		120
	435		105		135
	540		120		100
	420		90		72
	520		30		105
	415		60		160
	440		60		130
	550		165		75
	445		135		110
	430				130
					110
Series 2	460	Series 2	100	Series 2	60
	395		110		45
	490		105		120
	480		60		150
	415		130		75
	440		145		95
	435		80		110
	475		135		120
	490		140		130
	405		165		140
			120		150
			80		160
Average	474	Series 3	65		60
			85		45
			90		120
			65		150
			140		75
			56		95
			105		110
			80		120
			115		130
			115		140
			105		150
			125		160
			70		170
			150		180
			95		190
			65		200
			100		210
			180		220
			65		230
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verse direction, nor more than six to eight cell lengths removed from such necrotic tissue in a longitudinal direction. Photomicrographs illustrating these facts are shown in Figs. 12 and 13. On the other hand, permeability changes can be detected, in a large petiole, inches away from any sign of necrosis. The partial water-soaking of tissues which occurs near the discoloured zone is probably due to the pronounced modification of plasma membranes in this vicinity. It is not due to death of cells. Hence, it may be concluded that some factor other than pectinase activity contributed to the phenomenon of "action in advance" commonly attributed to soft rot pathogens, and, indeed, may be a necessary precursor to pectinase activity by which the tissues are finally disintegrated.

Permeability Changes Induced by the Dry Rot Fungus, Phoma lingam

Not only are the macroscopic symptoms of this type of disease markedly different from those of soft rots, but the protoplasts of infected cells also show an entirely different response to the presence of the pathogen. Not an

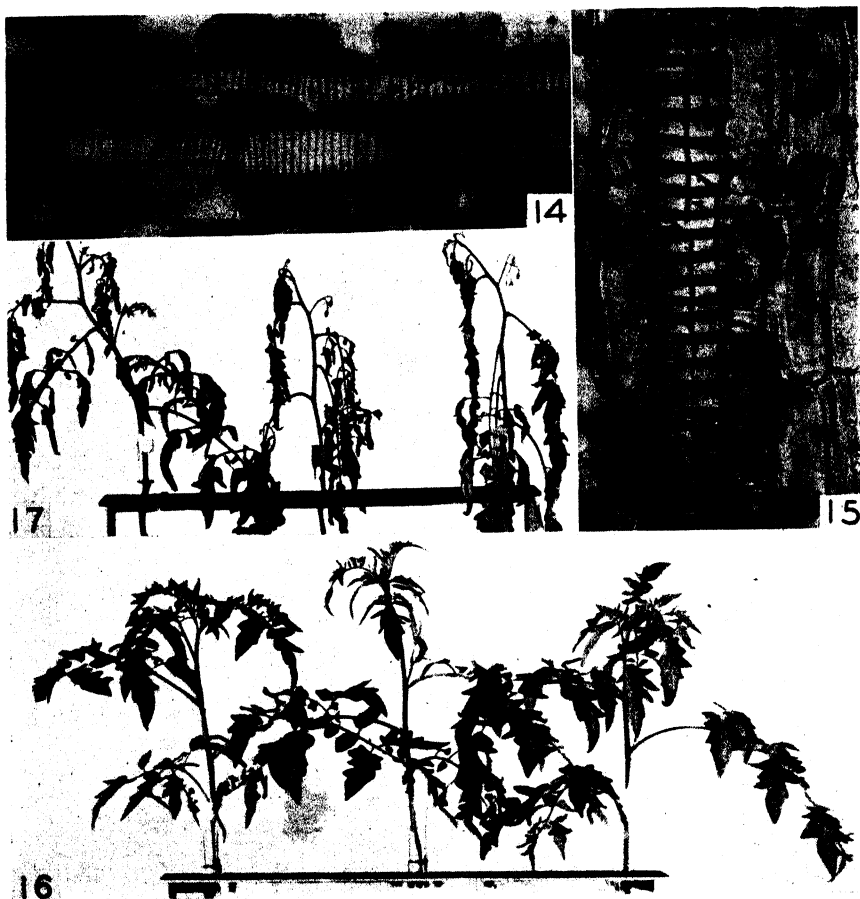
TABLE V

THE EFFECT OF *Phoma lingam* ON WATER PERMEABILITY OF XYLEM PARENCHYMA OF MATURE SWEDE "ROOTS"—PLASMOLYTIC METHOD

Healthy tissue		Tissue adjacent to necrotic	
No. of cells observed per section	Deplasmolysis time (sec.)	No. of cells observed per section	Deplasmolysis time (sec.)
15	100	7	225
3	110	2	265
1	90	5	260
10	100	2	270
10	80	3	320
1	55	1	200
5	85	1	210
1	80	6	240
16	90	7	270
1	100	8	280
5	80	1	300
3	100	5	320
1	60	1	350
1	90	10	380
1	65	10	360
3	75	4	240
1	120	1	300
5	80	5	270
1	110	10	320
1	115	5	270
1	125	4	240
1	45	2	320
10	70		
1	65		
1	95		
1	100		
Total	100	100	
Average	88		293



FIGS. 8 AND 9. Flecks on cleared leaves of Mindum wheat caused by *Puccinia graminis* *Tritici* race 36, showing extent of thallus development after 15 days. Minute sorus in Fig. 9. $\times 15$. FIG. 10. Mindum wheat with flecks caused by *P. graminis* *Tritici* race 36. (A) Untreated. (B) Narcotized. FIG. 11. Lesion in swede "root" caused by *Phoma lingam*; stained with Sudan IV and malachite green. $\times 80$ approx. FIGS. 12 AND 13. Longitudinal sections of celery petiole activated by *Botrytis*, stained with Ruthenium red; photographed with complementary filters to accentuate staining of pectin. $\times 120$ approx.



FIGS. 14 AND 15. Sections of living tissue cut $\frac{1}{4}$ cm. above end of excised tomato stems after wilting in filtrate from *Fusarium Lycopersici*. Stained with neutral red; plasmolysed in calcium chloride. Note living protoplasts of parenchyma cells in contact with tracheids. FIG. 16. Excised tomato plants immediately after placing in filtrates of cultures of *Fusarium Lycopersici*. FIG. 17. As in Fig. 16, but after standing 48 hr. in the filtrate.

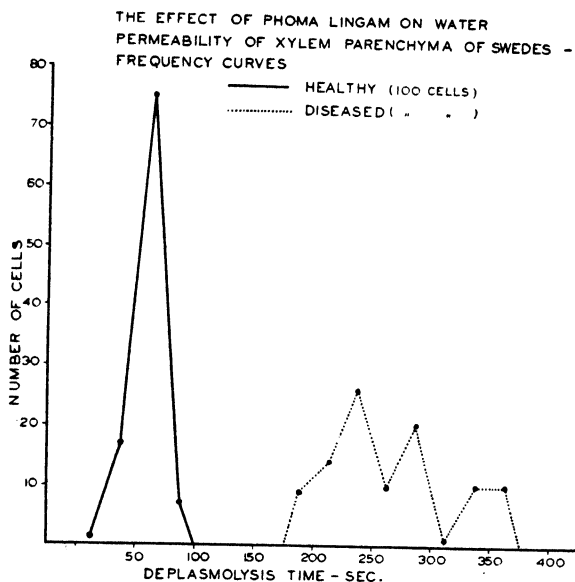


FIG. 18. The decrease in permeability to water of cells of swede root two to five cell diameters removed from tissue disintegrated by *Phoma lingam*. Cells plasmolysed in 2.0 calcium chloride; deplasmolysed in $\frac{1}{4}$ 0 calcium chloride.

increase, but a decrease in permeability is associated with this disease. Table V shows that *Phoma lingam* induces a threefold decrease in water permeability of cells in the outer xylem parenchyma of swede "roots" which are located from two to five cell diameters away from the clearly demarked necrotic zone that characterizes this dry rot disease. This symptom is indicated in Fig. 11. The frequency curves in Fig. 18, prepared from the data in this table, show this change very vividly. Every diseased cell of the 100 cells measured shows an emphatic decrease as compared with the cells obtained from precisely similar tissue zones of healthy tissue. It will be noted that the range in deplasmolysis time is considerably greater among the diseased cells. Factors jointly responsible for this are probably, (1) the presence of a permeability gradient across the zone in which the cells were measured, and (2) the unequal activation of the host protoplasts just as local differences in degree of disintegration of cell walls may be observed where such pathogenic change occurs.

This permeability decrease is not attributable to an apparent permeability change brought about by interference with diffusion of plasmolyte through the cell wall caused by a deposit of suberin or other fatty material in the wall. Sections treated with Sudan IV demonstrated the suberin of the normal periderm, but showed no sign of the presence of suberin in any other region. Fig. 11 is referred to as evidence for this statement. Accordingly, the observed permeability decrease may be safely considered as a change in true cytoplasmic permeability.

Permeability Changes Induced by *Phytophthora infestans*

The permeability changes induced by *Phytophthora infestans* are essentially similar to those reported above for the common soft rot fungi. In this instance permeability was measured with respect to both water and dextrose, since the large cylindrical cells in the cortical parenchyma of potato petioles are particularly adapted to measurement of permeability by plasmometric methods by which changes of permeability to a slowly penetrating substance like dextrose are most easily determined. (For details of this method see (34).)

TABLE VI

THE EFFECT OF *Phytophthora infestans* ON PERMEABILITY TO DEXTROSE OF CORTICAL PARENCHYMA CELLS OF POTATO PETIOLE—PLASMOMETRIC METHOD

Cell dimensions*											
Healthy tissue						Infected tissue					
Initial cell measurements			Cell measurements after time, <i>t</i> (= 17 hr.)			Initial cell measurements			Cell measurements after time, <i>t</i> (= 17 hr.)		
<i>L</i>	<i>l</i> _{min.}	<i>d</i>	<i>L</i>	<i>l</i>	<i>d</i>	<i>L</i>	<i>l</i> _{min.}	<i>d</i>	<i>L</i>	<i>l</i>	<i>d</i>
12	6.5	6	18	9	5	10	6	4	23.5	13.5	5
16	8	6.5	10	5.5	5	12	7	4	15	8.5	5.5
12	6	6	11.5	6	5	11.5	8	3	23	13	4.5
17	7.5	4.5	11.5	7	6	12	6.5	5.5	11.5	7.5	5.5
20	10	4	18	10	9	18	9	7	7.5	4.5	2
18	9	4.5	14	7	6	13	8.5	6.5	18	11	4
20	9.5	4.5	22	13	6	15	11	6	14	9	6.5
16	8	5	15	8	5	13	8	7	15	11.5	3
16	17.5	5.5	15	8.5	6	13	9	6	11	7	4
17	8	4	17	12	8.5	7	4.5	4	8	6	4
17	9	6.5	14	8	6	9	6.5	4.5	11	8	5
17	9	7	12.5	6.5	5	12.5	8	4	14.5	12	8
13	7	6	13	8	7.5	12	7.5	5.5	10.5	7.5	4.5
15	9	7	10	6	4.5	10	6	4.5	10	6.5	5
17	9	8.5	17	9	8	21	15	7	25	18	7
15	8	6	15	8	7.5	10	7.5	4.5	20	13.5	5
17	9	8	15	7.5	7	13	9	4	14	10	6
10	6	5.5	17	8	5	10	7.5	4.5	17	13	7
11	6.5	4	16	9	6	23	11.5	7	18	13	7.5
20	9	8	12	6.5	4	18	10	6	25	16	7
Average 15.8	8.6	5.9	14.7	8.1	6.1	13.1	8.3	5.2	15.5	10.4	5.3
Corrected values for <i>l</i>				8.7							

* Ocular micrometer units = 13.5 μ .

NOTE:

(1) Permeability: healthy tissue = 0.00001 millimols/cm.²/hr./mol.
infected tissue = 0.00019 millimols/cm.²/hr./mol.

(2) *L* = length of normal protoplast.

*l*_{min.} = original length of plasmolysed protoplast.

l = length of protoplast after time, *t*.

d = diameter of protoplast.

(3) The same cells as used in the first measurement could not always be recognized individually for the second measurement. Hence, *l* is corrected in the ratio of *L* (average) of the second group of measurements to *L* (average) of the first group.

Table VI presents the lineal measurements made individually on two groups of 20 cells of similar morphology obtained from several large petioles. One group comprised cells close to *Phytophthora* hyphae, and the other, cells from uninfected regions of the same petioles. Most of these latter cells were located on the side of the petiole opposite to that containing the hyphae and at the same relative distance from the leaf base.

The absolute permeability of healthy cells to dextrose is practically zero after 17 hr. The value presented, 0.00001 millimols/cm.²/hr./mol. concentration difference, is not significant, but a significant increase in dextrose permeability of the diseased cells is indicated by the value 0.00019 millimols/cm.²/hr./mol.

Infected plants were grown in an environment favourable to rapid disease development. Under such conditions a threefold increase in water permeability is noticed among the cells in tissues interspersed by mycelium one day after inoculation by infection with hyphal fragments. This increase is greater on the second day, but afterwards remains about constant for those cells near to the necrotic zone which becomes evident in a very short time. A degree of "action in advance" may, therefore, be attributed to this pathogen, just as has been described for soft rot fungi, since the plasma membrane is modified in advance of the mycelium, even though death of the cells does not occur in such a region. It is possible that the change noted on the first day was partly a response to the mechanical injury brought about by inoculation, but after the third day extensive destruction of tissue had occurred so that the permeability change established in advance of this necrotic region is most likely attributable to the presence of the pathogen (Table VII).

TABLE VII
THE EFFECT OF *Phytophthora infestans* ON WATER PERMEABILITY OF
INNER CORTICAL PARENCHYMA CELLS OF POTATO PETIOLE—
PLASMOLYTIC METHOD

Duration of infection (days)	Average time for deplasmolysis	
	Tissue interspersed with hyphae	Uninfected tissue
0	—	14 min. 10 sec.
1	4 min. 36 sec.	
2	2 min. 42 sec.	
3	3 min. 20 sec.	
4	3 min. 26 sec.	
5	3 min. 29 sec.	14 min. 50 sec.

Permeability Changes Induced by the Hadromycotic Fungus, Fusarium Lycopersici

Excised tomato plants placed in filtrates of Richard's medium in which *Fusarium Lycopersici* had been growing for four weeks became completely wilted within 24 to 48 hr. Photographs of some of these plants before and

after immersion in the fungus filtrate are shown in Figs. 16 and 17, respectively. A total of 20 plants responded in this way. Check plants placed in water or in the culture solution in which no organism had grown remained turgid and appeared quite normal. Plants would recover within two to three hours if the lower part of the stem were removed and the plants placed in water, provided wilting had not progressed too far. Permeability tests were applied to those leaves that were completely flaccid at time of sectioning, but that still contained a high proportion of living cells as indicated by neutral red staining and by their ability to plasmolyse in hypertonic calcium chloride solution.

The cells first measured, namely, the cylindrical, sparsely chlorophyllose cells lying near and parallel to the conducting tissue of the veins in the diseased leaves, show only a slight increase in permeability to water as compared with similar cells from healthy leaves. The average deplasmolysis time of the 315 healthy cells measured was 158 sec.; the average time for deplasmolysis of 315 cells from wilted leaves was 135 sec. (Because of the voluminous tables required, the individual data for this section of the work is not presented. It is recorded in an unpublished thesis (35).) However, the frequency curves presented in Fig. 19 indicate that a considerable proportion of the latter cells have suffered increase in permeability to a considerable degree. It will be noticed that the curves for both healthy and wilted cells show a secondary "peak". It is suggested that this was due to inclusion of cells of a functionally different tissue among those measured.

The permeability change of palisade cells is much more pronounced and is general among the cells of wilted tissue. Deplasmolysis times of 100 healthy and 100 diseased palisade cells are expressed as frequency curves also in Fig. 19. The average water permeability of palisade cells from wilted leaves is almost twice that of the healthy cells.

Because of the evident difference in degree of permeability change among cells in the lamina islets and those nearer the veins, the distribution of dead cells in badly wilted leaves was determined. Sections were cut completely across such leaves, stained in vital neutral red, and placed in slightly hypertonic calcium chloride solution. By this method dead cells are very easily distinguished from the living. Practically all protoplasts near the leaf margin were collapsed, and therefore dead. Cells nearest the midrib and larger veins were nearly all normal in appearance, though this varied somewhat with the time during which the test plants had been exposed to the action of the filtrate. The proportion of dead cells in central lamina tissue was intermediate between that of the margins and of the midrib regions.

Possible injury to tissues of the stem was then similarly investigated. Median and tangential longitudinal sections were cut from stems in a region about $\frac{1}{2}$ cm. above the cut end which had been exposed to the filtrate. These were then stained in vital neutral red and the cells plasmolysed in a hypertonic solution of calcium chloride. Immediate examination permits determination of cells already dead, and continued immersion for an extended period tends

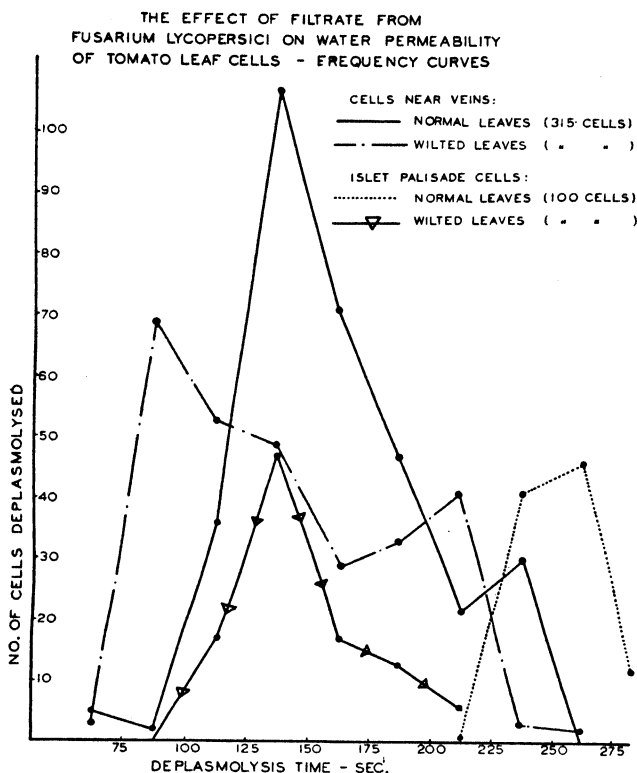


FIG. 19. The increase in permeability to water of two types of cells from excised tomato plants wilted after standing 48 hr. in a filtrate from cultures of *Fusarium Lycopersici*. Cells plasmolysed in 2.0 calcium chloride; deplasmolysed in $\frac{1}{4}$ 0 calcium chloride.

to bring about collapse of injured cells. Photomicrographs are shown of sections from two different stems after a 6 hr. immersion in a strongly hypertonic solution (Figs. 14 and 15). No evidence of abnormal death of cells in the vicinity of tracheids and vessels is to be seen. The living cells appear to be quite normal. This was general in all sections that had been cut from stems just a few hours after pronounced wilting had occurred. Cells of stems that had been allowed to remain in the filtrate for another three days were all dead, as was the entire plant.

Discussion

The hypothesis already elaborated to explain food uptake by obligate parasites gains additional support by the inclusion of *Puccinia graminis* and *Erysiphe Polygoni* in the list of obligate parasites known to cause an increase in permeability in susceptible hosts. The hypothesis might be invalidated to some degree if it could be demonstrated that the haustoria of the fungi examined were in direct contact with the vacuolar solution. The general consensus of opinion as revealed by Rice (26) in an extensive discussion of the subject

would seem to be that the haustoria invaginate the cytoplasmic layer of host cells and do not extend directly into the cell sap. Even though the haustorium were actually to penetrate the cytoplasmic layer, the fact that a new interface would be formed would probably lead to an aggregation of membrane forming materials at this interface. Investigation of this particular topic will be discussed in a paper to be published shortly.

If this hypothesis is a valid one, then it is to be expected that any factor modifying the permeability of plasma membranes of cells of a particular host would also tend to modify the susceptibility of that host, provided other changes that might be due to the same factor did not disturb the host parasite relationship sufficiently to offset this susceptibility change. The experiments with narcotics reported above fully justify this contention. Treatment with chloroform vapour increased the permeability of the plasma membranes of cells of *Mundum* wheat. Similar narcotization of inoculated plants of this variety resulted in an increased degree of susceptibility. The fact that the permeability of cells of a host resistant to a particular rust race shows a great local decrease as a response to rust invasion by this race, immediately points to one factor which militates against vigorous growth of the parasite and hence confers resistance to the host. Namely, when host cell permeability is decreased, availability of solutes to the parasite is similarly decreased. An extreme permeability change of this order might well result in inadequate nutrition and ultimate death of the parasite by starvation. This appears to support the contention, first succinctly propounded by Leach (18) for stem rust of wheat, and elaborated by Wellensiek (37) for corn rust, that resistance to rust is due to starvation of the parasite. A bulk of evidence which the author considers to be allied to this theory has since been presented, for discussions of which the reader is referred particularly to Gassner and Franke (12) and to Gassner and Hassebrauk (13).

The above findings might also be interpreted from the point of view of those investigators who subscribe to the "toxin-antitoxin" theory of rust resistance (5). Initial stimulation from the parasite may be considered to induce an active response on the part of the host, this response being expressed as a permeability decrease. This, in turn, may possibly be due to modification of the physical state of the protein in the plasma membrane, a change such as a partial dehydration of the protein-containing colloidal matrix tending to confer a reduced permeability to substances of the polarity of those tested by virtue of a reduction in pore size of the plasma membrane (7). Such a capacity for modification of the plasma membrane may be, in whole or in part, the particular "function of the living protoplasm of resistant species which prevents the development of the parasite", as stipulated by Chester (5, p. 293). However, the resultant effect of this change would still tend towards starvation of the parasite. As a secondary effect, an extreme decrease in permeability—such as was found—might well be a causal factor in the eventual death of the host cells themselves, which might explain the "flecking" well known as a symptom on resistant hosts. Any possible autotoxins ema-

nating from such dying cells, however, could operate against the parasite only some time after it had already been subject to lack of nutrients.

It could also be postulated that a toxin secreted by the fungus might kill those cells of a resistant host in which haustorium formation has been initiated, a conclusion reached by Allen (2) from histological studies, and that substances liberated from these dead cells might conceivably cause a decrease in permeability of neighbouring cells. Substances reported as being able to cause such a change are small concentrations of certain narcotics and some di- and trivalent cations. However, soft rot pathogens kill cells over a considerable zone some distance in advance of their mycelium, and a permeability change, which is probably indicative of some degree of injury, is also noticed in tissues beyond the killed zone. The change in this instance is an increase, so that it is improbable that death of the host cells referred to in the rust association can be responsible for the *decreased* permeability demonstrated. Whatever the cause of decreased permeability its net effect on the nutrition of the fungus remains the same, so that the experimental evidence presented above seems to interrelate the two main theories of rust resistance.

Referring now to the soft rot parasites *Botrytis* and *Sclerotinia*, it will be recalled that (1) the osmotic pressure of the fungus is greater than that of its host, (2) permeability of the host plasma membrane is increased, and (3) permeability change occurs in advance of those tissues affected by the characteristic pectinase activity of these fungi. The symptoms of celery decay caused by *Botrytis* and *Sclerotinia* are relatively constant. The writer has had opportunity to examine decaying celery in commercial cold storage plants and the symptoms noted in this environment are the same as developed after artificial inoculation by the method described. If petioles are inoculated near the upper end the brown discoloured region, indicative of necrosis, steadily progresses downwards. This region is always very moist and soft to the touch. A large supply of water is evident in this region. Meanwhile, however, the lower part of the petiole becomes dry and "pithy", which suggests that the fungus is in some way responsible for a flow of water from the lower uninfected parts of the petiole to its own locality. The fluid liberated from the killed cells will have a greater suction pressure than do the living cells. This follows directly from the relationship that suction pressure of a solution (or cell) is equivalent to osmotic pressure of that solution (or cell) less any other pressure that may be exerted upon it. In the case of the cell this latter factor is made up of wall pressure, which is a function of turgor, so that its suction pressure is correspondingly increased. Accordingly, water is drawn from uninfected regions. This effect may be accentuated by loss of water from the fluid suffusing the killed cells due to evaporation, and also by increase in osmotically active solutes as a result of hydrolysis of pectins occurring in this region and of hydrolysis of sap substances by enzymes liberated from disorganized cells (as occurs in expressed sap). The much greater osmotic value of the fungus enables it to absorb water from this solution, this explanation indicating why a parasite invariably has a higher

osmotic pressure than its host. In addition, some solutes are able to diffuse from the living cells when permeability is modified, and this would ultimately get into the free sap in the vicinity of the fungus, where all assimilable solutes are available to the fungus. Some such accessory activity seems probable since pectin hydrolysis can satisfy only the carbon requirement of the parasite. Nitrogen and the other essential elements must be obtained elsewhere. To a large extent they are doubtless provided by killed cells, but some other hypothesis such as that presented above is necessary to explain the ability of these and allied fungi to satisfy their food requirements during the initial period before pectin hydrolysis has reached completion and death of protoplasts has occurred.

It seems likely that this accessory nutritive function of permeability change may be of even greater importance in the parasitism of organisms such as *Phytophthora infestans*, which, though it is virulent in its disease inducing propensities, nevertheless grows very poorly or not at all on synthetic culture media. Because this organism will not make thrifty growth on dead plant tissue, it seems reasonable to suggest that it is aided in its parasitism by substances obtained from living cells, which the demonstrated host cell permeability increase that extends even to dextrose, might make possible. Support for this suggestion is given by the fact that the distal parts of the *Phytophthora* mycelium remain in regions of living host tissue.

The permeability change effected in palisade cells by the powdery mildew *Erysiphe Polygoni* is not very pronounced (Fig. 6), though it is definite. The examination of this tissue, which is not actually invaded by the parasite, was undertaken in order to determine any possible relationship that might exist between permeability change and increased respiration as induced by powdery mildews. Yarwood (39), Pratt (24), and Allen and Goddard (1) have shown that leaf infection by powdery mildews has resulted in a strongly increased respiration rate. Allen and Goddard were able to show that the respiration of the host tissue is greatly increased by mildew, over and above the increase caused by the respiration of the mildew itself, a 4 to 1 increase being reported. The effect of infection is confined to the tissues immediately underlying the infected area. The above authors suggest that a possible change in protoplasmic structure may increase oxygen consumption by permitting greater activity of carbohydrate hydrolysing enzymes. Such a protoplasmic change might conceivably involve a modification of permeability of the cytoplasm as has been indicated. This would tend to increase transpiration rate—Lachenmeier (17) and Singh and Das Gupta (28) have related change in permeability of leaf mesophyll with change in transpiration rate—which tends to modify other metabolic processes (9). Hence, there seems to be some evidence of interrelation between changes of the three host factors, permeability, transpiration, and respiration, as influenced by parasitism. However, the writer makes this suggestion with considerable reserve because the permeability change demonstrated experimentally does not seem to be

sufficiently pronounced to warrant much stress being placed at present upon its importance in the instance of the mildew studied.

The decrease in permeability found to be associated with a dry rot disease was entirely unexpected, and explanation for its cause and effect is largely a matter for conjecture. That the change is of cytoplasmic origin and not due to cell wall impregnation with suberin was clearly demonstrated by the suberin tests reported above. The principal activity of the fungus seems to be a cellulose decomposition, since, after using the ruthenium red test, pectin in the middle lamella was more readily discerned among the disintegrating, collapsed cell walls than in normal tissues, but whether the decreased permeability of cells near the dead tissue is a change induced directly by this disintegrative activity of the fungus or is produced as an active response, it is not possible to say. If the latter is true, the decreased permeability may be interpreted as a protective action by the host in setting up a barrier against the fungus, the zone of low permeability tending to prevent solutes and water from reaching the region occupied by the fungus. This would be in keeping with Brown's (4) suggestion that ability of the host cells to withhold water from the parasite determines a dry rot by arresting the activity of fungal enzymes at an intermediate stage. Virulent soft rot organisms are able to overcome this protective action by virtue of their ability to cause an increased permeability, as has been discussed.

The permeability changes that have been shown to occur in the leaf cells of plants subjected to the products of a hadromycotic fungus certainly clarify the previous state of conjecture relative to the possibility of such changes, but the uncertain state of knowledge of the factors controlling normal transpiration and translocation throws some doubt on the accuracy of conclusions that may be drawn from such results.

No fully adequate concept of the precise cause of wilting as found in hadromycoses has been elaborated. As Rudolph (27) pointed out, the various theories that have been forwarded to explain the wilting caused by vascular parasites fall into two main categories: (1) wilting is caused by some form of vascular obstruction in which possible obstructing agencies are the parasitic mycelium, gums formed during disease development, embolism products such as carbon dioxide, and disease induced tyloses, all of which may tend to prevent an adequate supply of water reaching the leaves; (2) wilting is caused by the injurious action of toxins arising either from the fungus or from cells killed by it, and translocated from the site of infection to the leaf mesophyll, so that affected leaf cells are unable to control their rate of water loss. Clayton (6) suggested that any such toxin would be expected to exercise its deleterious effect by modifying the water permeability of the plasma membrane of the mesophyll cells, an increase in permeability allowing greater mobility of water and consequent increased transpiration, or else a decrease in permeability preventing cells removed from the veins from receiving an adequate supply of water. Fisher (11) refers to the same suggestion. No measurements of permeability are presented by either worker. The same concept suggesting

permeability change as contributive to pathological wilting is expressed by Dixon (10), who stated that wilting of "poisoned" plants was due to the modification of the turgor properties of mesophyll cells brought about by the "poisonous substances rendering the cell membrane permeable". Linford (21) concluded from studies of the transpiration of pea plants infected with *Fusarium orthoceras* var. *pisi* that the plants were injured because of excessive transpiration resulting from a toxin induced modification of the mesophyll cells. Bakke (3) found a sudden increase of transpiration to a maximal rate at the onset of permanent wilting in droughted plants, an increase that he ascribed to rupture of the water columns of the xylem. This increase is followed by a decrease as the wilted plant dries out.

The "toxin" hypothesis interpreting the wilting caused by vascular parasites receives strong support by comparison with results from several studies concerned with the ascent of sap in which wilting resulting from mechanical injury to roots or stem has been explained as due to a deleterious effect of toxins liberated from killed cells. Such toxins were considered to have effect upon either the cells of the leaf or the living cells of the xylem (36, 22, 10, 16, 23). Peirce (23) stated that living cells associated with the non-living elements in the xylem exert an essential conditioning influence on the flow of water, and "when by reason of cold, or heat, or poison, they [the living cells] fail to maintain the system, the vascular tissues fail to perform properly." Herein is another tenable hypothesis of a contributory cause of wilting of parasitic origin, though it is possible that the necessary "condition" may be absence of dead cells rather than the presence of living ones, since exudate from dead cells leads to pit closure.

Still a further suggestion may be made to the effect that wilting may in some instances be caused by a toxic action of an excessive accumulation of specific ions or by the entry into the plant of ions normally excluded by the permeability mechanisms of the root cortex which Curtis (9, p. 78) states may occur if a pathogen causes injury to these latter tissues. This suggestion is based on the statement by Strasburger (33) that the living cells between the root surface and the xylem greatly influence entry of solutes into the plant, and that when the differentially permeable membranes in this region were killed, any solute could be taken up and many substances could be absorbed much more rapidly than through living roots. Subsequent amplification of the former part of this statement has been referred to by Crafts and Broyer (8).

The experiments with stems of wilted excised plants, reported herein, prove that the typical symptom may be induced without any killing of normally living cells associated with the conducting elements. Pit closure is another possibility, but, in the stems examined, the bulk of the xylem was of primary origin so that pit closure could be only of small importance, and, in any case, no cell disintegration was observed which would free the colloidal material supposedly causing this obstruction. The application of extract to ends of cuttings eliminates ion selection by roots in this particular experiment. Hence, the foregoing evidence suggests that the factor directly responsible

for wilting is primarily active in the leaves, and is doubtless a product of fungus origin.

The statement made by Clayton (6) relative to the possible importance of permeability change in the leaf mesophyll needs further examination. A stipulated possible decrease in permeability need not be considered because a decrease does not occur. The idea was presented to the writer that permeability increase would tend to militate against local injury, such as occurs first at the leaf margins, by allowing a more speedy replacement from other cells of the water transpired from a particular mesophyll region. However, the results presented show that permeability increase is greatest in the mesophyll regions farthest removed from veins, so that in these latter regions increased transpiration cannot be offset by a more ready availability of water from cells nearer the source of supply because of the difference in degree of permeability change which each region undergoes.

Dixon (10) and Knight (15) have demonstrated that the water balance in leaves is an extremely delicate one. The latter author found that a loss of only 1% of the leaf water content would cause some leaves to reach a flaccid condition, during conditions of drought. Hence, under conditions that tend to induce a flaccid condition of leaf cells, only a small additional tax upon the water supply could bring about wilting to an injurious degree. The work of Lachenmeier (17) and Singh and Das Gupta (28) indicates that increased permeability would bring about this additional demand on the water in the vessels, since each of these workers relates change of plasma permeability with transpiration rate, and Das Gupta states, ". . . in the absence of . . . other controlling factors . . . , increased or decreased transpiration may well be explained on the basis of permeability changes in the protoplasm of mesophyll cells."

Thus, from the evidence at hand, the following sequence of events seems the most probable. The presence in the leaves of a metabolic product of a hadromycotic fungus causes an increase in permeability to water which is most pronounced where accumulation of the fungal irritant occurs most extensively. This increase in permeability promotes more rapid transpiration, and the consequent additional demand for water upsets the delicate water balance of the plant, and, under conditions normally nearly limiting for maintenance of turgor, causes progressive wilting, which finally becomes permanent and leads to death of the mesophyll cells.

The suggestion that the fungal irritant accumulates in the mesophyll needs amplification, since, if its method of movement across living tissues were based on simple diffusion through cells, local accumulation in the mesophyll would require the supposition of some mechanism of fixation or precipitation in these tissues. The suggestion is therefore offered that movement of the toxin from the tracheids may be by way of a flow of solution along the cell walls or intercellular spaces. Evaporation of water then permits solute concentration. The writer has found histopathological evidence that fungal

toxins do move through parenchyma tissue, in some instances, in such a fashion.

An alternative suggestion to explain wilting was made in personal correspondence by Dr. G. W. Scarth. As a result of the increased permeability of mesophyll protoplasts a slow leaching out of solutes may cause loss of turgor, which if maintained, would be followed soon by death. With approaching death a drastic increase in permeability is to be expected with consequent sudden, extensive water loss.

The possibility that permeability increase is merely a preliminary indication of a toxic action which would ultimately prove lethal without intervention of wilting is at least partly offset by the fact that recovery from wilting, up to a certain degree of severity, has been found to occur.

Acknowledgments

The author wishes to express thanks for the guidance and for the many suggestions given so pertinently by Professor J. G. Coulson, Professor of Plant Pathology, Macdonald College, McGill University, and by Dr. G. W. Scarth, Professor of Botany, McGill University, who were codirectors of this study.

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CLASSIFICATION REVISION IN *XANTHOMONAS TRANSLUCENS*¹

By W. A. F. HAGBORG²

Abstract

The species description of *Xanthomonas translucens* (J. J. and R.) Dowson is emended to include five closely related organisms, which are distinguishable chiefly by differences in pathogenic capabilities on wheat, oats, barley, and rye. One of these organisms, *X. translucens* (J. J. and R.) Dowson, is given new rank as *X. translucens* f. sp. *hordei*; another, *Phytomonas translucens* f. sp. *undulosa* (S. J. and R.) Hagborg, is transferred to the genus *Xanthomonas*; still another, *Phytomonas translucens* var. *secalis* (R. G. and J.) Bergey *et al.*, is given new rank and also transferred to the genus *Xanthomonas* as *X. translucens* f. sp. *secalis*; the remaining two, *X. translucens* f. sp. *hordei-avenae* and *X. translucens* f. sp. *cerealis*, are described for the first time.

The results of non-determinative comparative studies in physiology and serology of the second and last two of the above-mentioned special forms of *Xanthomonas translucens* (J. J. and R.) Dowson emend. are given; these show their close similarity in characters other than pathogenicity. These studies also show the presence of some variation between different isolates of the same special form. Parallel studies, made at the same time, on cultures of *Pseudomonas atrofaciens* (McC.) Stapp, *P. coronafaciens* (Elliott) Stapp, and *P. medicaginis* var. *phaseolicola* (Burkh.) Stapp and Kotte, were used as controls on the methods.

Introduction

The need for some revision of the classification of *Phytomonas translucens* has long been apparent. In 1917, Jones, Johnson, and Reddy (21) described the species as *Bacterium translucens*, the organism causing bacterial blight of barley. Two years later, Smith, Jones, and Reddy (25) described a variety, *B. translucens* var. *undulosum*, which was like the original species, except that it was capable of attacking wheat, barley, and rye. In 1924, Reddy, Godkin, and Johnson (24) described a second variety, *B. translucens* var. *secalis*, which was essentially like the other two, except that it was capable of attacking rye only. In 1936, Hagborg (18) transferred *B. translucens* var. *undulosum* to the genus *Phytomonas* Bergey *et al.* and changed its rank to that of a *forma specialis*, in conformity with the International Rules of Botanical Nomenclature of 1930. In 1939, Dowson transferred the original species to his newly created genus *Xanthomonas*, as *X. translucens* (J. J. and R.) Dowson (10). Since then, the writer has found two additional parasitically specialized forms of the same species, which are here reported. He accepts Dowson's genus *Xanthomonas*, but emends the description of the species *X. translucens* to include all the derivatives, which are then described as five *formae speciales* of the emended species. The results of detailed physiological and serological studies with several pure cultures of three of these forms are given to show the degree of similarity that exists among them.

¹ Manuscript received in original form October 25, 1941, and as revised, March 19, 1942.

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Methods

For studying the pathogenic capabilities of isolates, a method was used that has been described previously (18) and that permits the testing of a large number of isolates on the four cereal hosts with a minimum of labour. This method consists essentially in wounding the primary leaf with a flamed, nichrome needle that has been cooled and dipped in the inoculum before piercing the leaf tissues. From 10 to 15 seedlings of each host were inoculated with each isolate. The plants were then held at a greenhouse temperature of about 25° C. for 10 days, when they were examined for infection. In the event of a positive result every leaf, as a rule, becomes infected. Check plants show only clear-cut wounds with no infection around the margins.

For all bacteriological tests, unless otherwise indicated, the methods used are those recommended by the Committee on Bacteriological Technic of the Society of American Bacteriologists, as given in the Manual of Methods for Pure Culture Study of Bacteria (9). Flagella were stained by the method of Casares-Gil, endospores were sought by the Schaeffer and Fulton modification of the Wirtz method, and gelatine liquefaction was studied by the incubation of inoculated plain gelatine at room temperatures. The reduction of nitrate to nitrite in peptone beef broth and agar was tested by the α -naphthylamide-sulphanilic acid method, and the presence of unreduced nitrate following incubation was proved by the appearance of a red colour after the addition of zinc dust. Gram staining was done according to the method of Kopeloff and Beerman (22). For the development of a water soluble, green, fluorescent pigment, the cultures were tested in the more concentrated of the two modifications of Sullivan's solution, as recommended by Clara (8). Hydrogen sulphide production was studied by the test strip technique of Zo Bell and Feltham in a culture medium consisting of peptone beef broth containing 0.2% magnesium sulphate. For the detection of ammonia, three drops of Nessler's reagent were added to the lower half of the cotton plug of the culture tube, after incubation for two weeks, and the plug replaced for one minute before the colour was recorded.

For the carbohydrate cleavage studies Durham fermentation tubes were used. All carbohydrates were tested as a 1% solution or suspension in the inorganic basal medium of Ayers, Rupp, and Johnson, as modified by Conn *et al.* (9), with brom cresol purple added as an indicator of hydrogen ion concentration. Most of the carbohydrate media were sterilized by autoclaving for 18 min. at 15 lb. steam pressure, but the sugars levulose, sucrose, lactose, and raffinose were sterilized in concentrated solution by filtration and then added to a suitably concentrated solution of the autoclaved basal medium. Media that were sterilized by filtration were held at least four weeks at incubation temperature to permit the discarding of any contaminated tubes before inoculation. Observation of acid production was made after incubation periods of one month and two months, the pH being determined by means of a Hellige disc comparator.

In the test for starch hydrolysis a departure was made from the method recommended in the Manual of Methods for Pure Culture Study of Bacteria (9). The recommended method consists in the use of a saturated aqueous solution of iodine, but, as first shown by Mylius (23), the starch iodine test is unreliable except in the presence of hydriodic acid or an iodide salt. Because the special forms of *X. translucens* cause an alkaline reaction in beef peptone starch broth, due to ammonia production, it is necessary with these organisms to acidify the medium before testing with iodine. Without acidification, hydrolysis, as indicated by a negative test for starch, appears to be present even where there is none. The justification of acidification was demonstrated by testing the uninoculated starch broth after it had been rendered slightly alkaline with ammonium hydroxide. The starch iodine test with such broth was negative, but, with acidification before the addition of iodine, the test was positive.

The incubation temperature with all media except gelatine was maintained at 26° to 28° C. With gelatine it was somewhat lower (20° to 24° C.). Frazier's method (13) of demonstrating gelatine decomposition gave fully confirmatory results at 26° to 28° C.

For serological studies, a serum capable of agglutinating *X. translucens* f. sp. *undulosa* was prepared. A sample of the blood of a half-grown rabbit was tested for agglutination properties with *X. translucens* f. sp. *undulosa*, culture No. 385. No agglutination occurred with this serum even at a concentration of 50%. The rabbit was then inoculated three times, intraperitoneally, at three-day intervals, with 1 ml., 2 ml., and 3 ml. portions of suspensions of young cultures of *X. translucens* f. sp. *undulosa*, Culture 385, in physiological salt solution. A test sample, drawn one week after the last inoculation, caused agglutination of the organism at a final serum concentration of 2.5%. Exactly one month after the last inoculation, the rabbit was bled and the serum in a concentration of 2% was found to be quite effective in 15 min. at about 25° C. Preservation of the serum¹ by the addition of phenol to a concentration of 0.5% did not impair its usefulness. This serum was diluted with phenolated physiological salt solution to the desired concentrations. The dilutions selected were the same as suggested by Conn *et al.* (9). The cultures to be tested were increased on beef peptone agar, then suspended in phenolated saline. The concentration of each suspension was adjusted to equal, in light transmitting properties, a precipitate of barium sulphate made by adding barium chloride to a 1.0% sulphuric acid solution to a concentration of 10^{-3.5}. The macroscopic agglutination test was made by mixing equal parts of the serum dilution in serological tubes and holding them overnight in an oven at 50° C.

Sources of Cultures

The numerous cultures used in these studies were obtained in the course of an investigation made by the author to determine what pathogens were

¹ Limited quantities of this serum, designated serum A, can be supplied to investigators who require it.

concerned in the bacterial diseases occurring on cereals in Canada. Isolations were commenced in 1932 and continued through 1940, the chief source of material being specimens of bacterial diseases and diseases suspected of being bacterial in origin, which were collected on annual surveys in Manitoba. These collections were supplemented by others kindly forwarded from other parts of Canada, chiefly from eastern Canada and the province of Saskatchewan. In all, 373 collections consisting of wheat, oats, barley, rye, and *Bromus inermis* Leyss. were studied.

The pathogens isolated from the disease lesions included several species and varieties, namely, *Pseudomonas atrofaciens*, *P. coronafaciens* and organisms closely similar to it, *P. coronafaciens* var. *atropurpurea* (R. and G.) Stapp, *X. translucens* f. sp. *undulosa* (S. J. and R.) comb. nov., *X. translucens* f. sp. *secalis* (R. G. and J.) comb. nov., and the two new *formae speciales* described below. The distribution of each of the pathogens on the different hosts was reported previously (19), at which time one of the new *formae speciales* of *X. translucens*, described below as f. sp. *hordei-avenae*, was included under *Phytomonas translucens* (J. J. and R.) Bergey *et al.*

The various *formae speciales* of *X. translucens* were found attacking wheat, barley, and rye, but not oats. Each occurred chiefly on the cereal originally described as its host in nature. Of 22 collections of bacterial infection on barley, every one yielded either the barley blight organism, *X. translucens* f. sp. *hordei* or the new f. sp. *hordei-avenae*. *X. translucens* f. sp. *undulosa* was found in 83 collections of wheat and in one collection of rye, and *X. translucens* var. *secalis* in only one collection of rye. As rye is only grown to a limited extent in the area surveyed, the fields available for examination were relatively few in comparison with those of the other three cereals.

Nomenclature

Special classifications, as defined recently by Gilmour and Turrill (15), are particularly useful in dealing with bacteria. They can often meet the needs of special fields of investigation where the use of a general classification, based on a maximum correlation of attributes, is impractical or inadequately developed. Among the bacterial plant pathogens, special emphasis needs to be attached to infection capabilities, hence the need of a descriptive category based on pathogenicity. To meet this need the subdivision of species into *formae speciales* seems particularly well suited.

As some authors have adopted the category *formae speciales* and others have not, it seems desirable to state the case for its use. Adoption of this designation was recommended at the Fifth International Botanical Congress, 1930. Recommendation I of the International Rules of Botanical Nomenclature, adopted at that congress, reads as follows:

"In parasites, especially parasitic fungi, authors who do not give specific value to forms characterized from a biological standpoint, but scarcely or not at all from a morphological standpoint, should distinguish within the species special forms (*forma specialis*) characterized by their adaptation to different hosts."

Some doubt may exist as to whether or not this recommendation was meant to be retroactive, but it is to be hoped that the recommendation may be considered retroactive where named organisms are obviously of the same rank as new *formae speciales*. As one of the primary purposes of the International Rules of Botanical Nomenclature is to avoid names that may "throw science into confusion", it is believed that the revision recommended in the present paper is in keeping with the spirit of those rules.

The Species *Xanthomonas translucens*

It has been found necessary to broaden, slightly, the species description of *Xanthomonas translucens*, as given originally by Jones, Johnson, and Reddy, in order to include in it the rye organism, var. *secalis*, which does not attack barley. The ability to attack barley is one of the characteristics given in the original description.

ORIGINAL DESCRIPTION

The original species description is as follows:

"*Bacterium translucens* n. sp.

"Cylindrical rods rounded at ends, solitary or in pairs; individual rods 0.5 to 0.8 by 1 to 2.5 μ , motile by a single polar flagellum; aerobic, no spores.

"Superficial colonies in peptone-beef agar plates round, smooth, shining, amorphous except for inconspicuous somewhat irregular concentric striations within, wax-yellow tinged with old-gold; margin entire.

"Liquefies gelatin slowly; produces slight acidity in milk; digests casein; nitrates not reduced; acid produced in cultures with various sugars. No gas produced. Gram-negative. Group number 211. 222532.

"Pathogenic in leaves of *Hordeum vulgare*, *H. distichum*, *H. hexastichum*, forming translucent elliptical to striaform lesions.

"Type locality: Madison, Wis., on *Hordeum vulgare*.

"Distribution: Northern Mississippi Valley and westward to Pacific coast."

In the emended species description, given below, all characters not essential to the determination of the species under the system employed by Bergey *et al.* (4) have been omitted, but are dealt with as non-determinative characters in subsequent sections. One character employed by Bergey in differentiating species, namely, the ability to hydrolyse starch, did not appear in the original paper by Jones, Johnson, and Reddy. They stated that there is "no evidence of diastatic action on potato starch suspended in peptone-beef agar, tests being made with potassium-iodid-iodin". Similarly Godkin (16) found "no reduction of starch" with either the barley organism or f. sp. *undulosa*. More recently Dowson (10) reported hydrolysis of starch by a culture of *Phytomonas translucens* that was sent to him by Dr. W. L. Waterhouse, of Sydney, Australia. As experiments, given below, suggest that the organism is weak in the production of diastatic enzymes and that the confusions in the literature may be due to differences in method (e.g., length of incubation) it has not been deemed desirable to add the ability to hydrolyse starch to the species description. This character is treated below in a separate section.

DETERMINATIVE DESCRIPTION OF *X. translucens* (J. J. AND R.) DOWSON
EMEND.

Straight rods, not producing endospores, motile by a single polar flagellum.

Growth on peptone beef agar yellow after four days. Gelatine liquefied, nitrite not produced from nitrate. Pathogenic in one or more genera of the family *Gramineae*. Known to consist of several *formae speciales*.

The Special Forms of *X. translucens*

NEW SPECIAL FORMS

The two new *formae speciales* of *X. translucens*, mentioned above, unlike any of the forms previously reported, are both capable of causing water-soaked lesions on oat seedlings following artificial inoculation. Inoculation may be made either with or without wounding. Like *X. translucens* f. sp. *hordei*, they consist of straight, yellow rods, which are motile by means of a single polar flagellum, they do not produce endospores, they liquefy gelatine, and they are incapable of reducing nitrate to nitrite. One of them can infect barley and oats, but not wheat and rye, and is here named *Xanthomonas translucens* f. sp. *hordei-avenae*; the other can infect wheat, oats, barley, and rye, and is here named *Xanthomonas translucens* f. sp. *cerealis*.

DETERMINATIVE DESCRIPTION OF KNOWN SPECIAL FORMS OF
Xanthomonas translucens

1. *Xanthomonas translucens* f. sp. *hordei* f. sp. nov.

Synonymy: *Bacterium translucens* Jones, Johnson, and Reddy, 1917, *sensu stricto*.
Pseudomonas translucens Stapp, 1928.
Phytomonas translucens Bergey *et al.*, 1930.
Xanthomonas translucens Dowson, 1939.

Occurs naturally on *Hordeum* spp. Produces water-soaked infections, following wound inoculation, at 25° to 30° C. in seedlings of *Hordeum* spp., but not of *Triticum* spp., *Avena* spp., or of *Secale cereale*.

2. *Xanthomonas translucens* f. sp. *undulosa* (S. J. and R.) comb. nov.

Synonymy: *Bacterium translucens* var. *undulosum* Smith, Jones, and Reddy, 1919.
Pseudomonas translucens var. *undulosa* Stapp, 1928.
Phytomonas translucens f. sp. *undulosa* Hagborg, 1936.

Occurs naturally on *Triticum* spp. and on *Secale cereale*. Produces water-soaked infections, following wound inoculation, at 25° to 30° C. in seedlings of *Triticum* spp., *Hordeum* spp., and of *Secale cereale*, but not of *Avena* spp.

3. *Xanthomonas translucens* f. sp. *secalis* (R. G. and J.) comb. nov.

Synonymy: *Bacterium translucens* var. *secalis* Reddy, Godkin, and Johnson, 1924.
Pseudomonas translucens var. *secalis* Stapp, 1928.
Phytomonas translucens var. *secalis* Bergey *et al.*, 1939.

Occurs naturally on *Secale cereale*. Produces water-soaked infections, following wound inoculation, at 25° to 30° C. in seedlings of *Secale cereale*, but not of *Triticum* spp., *Hordeum* spp., or of *Avena* spp.

4. *Xanthomonas translucens* f. sp. *hordei-avenae* f. sp. nov.

Occurs naturally on *Hordeum* spp. Produces water-soaked infections, following wound inoculation, at 25° to 30° C. in seedlings of *Hordeum* spp. and *Avena* spp., but not of *Triticum* spp., or of *Secale cereale*.

5. *Xanthomonas translucens* f. sp. *cerealis* f. sp. nov.

Occurs naturally on *Triticum* spp. Produces water-soaked infections, following wound inoculation, at 25° to 30° C. in seedlings of *Triticum* spp., *Hordeum* spp., *Avena* spp., and of *Secale cereale*.

Non-Determinative Comparative Studies

The non-determinative cultural characters of 13 pure cultures of *X. translucens* emend., of monocolonial origin, which had been isolated from various cereals and found pathogenic on one or more of them, were studied in detail. The purposes of these comparative studies were, (1) to determine the extent of the similarity between the special forms, (2) to find if any cultural characters were correlated with pathogenic capabilities, and (3) to find the degree of variation, if any, between different isolates of the same special form.

The cultures chosen were derived from collections made in several different years and in several different localities in order to increase the chances of observing variations between different isolates of the same special form. All cultures were either recently isolated or recently reisolated, before commencement of the comparative studies. All were tested for each of the species characters and found to conform to the emended description as given above. For convenience, these 13 pure cultures will be referred to as the test cultures. For purposes of comparison, pure cultures of *Pseudomonas atrofaciens*, *P. coronafaciens*, and *P. medicaginis* var. *phaseolicola* were included in the studies. These cultures will be referred to as the check cultures. A historical summary of information on the pure cultures included in the studies is given in Table I.

Hydrogen Sulphide Production

All the test cultures produced hydrogen sulphide by the sixth day after inoculation, but no hydrogen sulphide was produced by any of the check cultures even after incubation for 29 days.

Ammonia Production

All the cultures studied, whether test or check cultures, produced ammonia in peptone beef broth. Uninoculated control tubes gave negative results until held over ammonium hydroxide.

Green Pigment Production

A water soluble, green, fluorescent pigment was not produced by any of the test cultures. *Pseudomonas atrofaciens* produced such a pigment visible in diffuse daylight after three days. *P. coronafaciens* and *P. medicaginis* var. *phaseolicola* produced a substance that gave a green fluorescence only when observed under ultra-violet light in a dark room.

Changes in Litmus Milk

All the test cultures reduced the litmus fairly rapidly, following the production of a slight alkalinity. No litmus reduction occurred with the check cultures, which caused a distinctly alkaline reaction. One week after inoculation the test cultures had caused almost no change in the medium, but all the check cultures had caused the medium to turn bluish. After one month, the test cultures were light brown, those of *P. atrofaciens* and *P. coronafaciens* were dark brown and semitranslucent, and those of *P. medicaginis* var. *phaseolicola*, blue-grey and opaque. The check cultures turned red on the addition of acid, but the test culture tubes remained unchanged following

TABLE I

DATA RELATIVE TO SOURCE, YEAR OF COLLECTION, ORIGINAL HOST, CULTURAL HISTORY, AND PATHOGENICITY IN SEEDLINGS, OF THE PURE CULTURES INCLUDED IN THE NON-DETERMINATIVE COMPARATIVE STUDIES

Accession number of pure cultures	Locality of origin of collection (Manitoba)	Year of collection	Original host	Cultural history	Pathogenicity in seedlings			
					Wheat	Oats	Barley	Rye
<i>X. translucens</i> f. sp. <i>undulosa</i>								
191	Brandon	1933	Wheat	M.C.	+	0	+	+
385	Winnipeg	1934	Wheat	R.M.C.	+	0	+	+
473	Oak Lake	1935	Wheat	R.M.C.	+	0	+	+
481	Bowsman	1935	Wheat	R.M.C.	+	0	+	+
618	Neepawa	1936	Wheat	R.M.C.	+	0	+	+
884	Swan River	1938	Wheat	R.M.C.	+	0	+	+
<i>X. translucens</i> f. sp. <i>hordei-avenae</i>								
239	Fannystelle	1933	Barley	R.M.C.	0	+	+	0
349	Brandon	1934	Barley	R.M.C.	0	+	+	0
377	Morden	1934	Barley	R.M.C.	0	+	+	0
451	Brandon	1935	Barley	R.M.C.	0	+	+	0
1011	Winnipeg	1939	Barley	R.M.C.	0	+	+	0
1174	Winnipeg	1940	Barley	M.C.	0	+	+	0
<i>X. translucens</i> f. sp. <i>cerealis</i>								
1027	Virden	1939	Wheat	M.C.	+	+	+	+
<i>P. atrofaciens</i>								
909	Winnipeg	1938	Wheat	R.M.C.	<i>m</i>	<i>m</i>	0	<i>m</i>
<i>P. coronafaciens</i>								
1014	Winnipeg	1939	Oats	M.C.	<i>hm</i>	<i>+h</i>	0	<i>h0</i>
<i>P. medicaginis</i> var. <i>phaseolicola</i>								
1090	Winnipeg	1939	Wax beans	M.C.	0	0	0	0

NOTE—Meaning of symbols: R.M.C. = a culture of monoclonal origin, reisolated from a pure culture infection. M.C. = a culture of monoclonal origin isolated from an original field collection. 0 = no visible infection. + = water-soaked infection. m = brown to black margins around wounds. h = chlorotic halo around wounds.

the addition of either acid or base. This indicated decomposition of the litmus by the test cultures only. The uninoculated control tubes remained unchanged in colour throughout the test.

Carbohydrate Cleavage

Jones *et al.* (21), Godkin (16), and Bamberg (2) made studies on carbohydrate cleavage by one or more of the special forms of *X. translucens*, but all of these authors used peptone in the basal medium. As all the organisms studied produce ammonia in the presence of organic nitrogen, the inconsistent results of these investigators are thus explained. For organisms that produce an alkali in proteinaceous media, Ayers, Rupp, and Johnson (1) have suggested the use of nitrogen in inorganic form only. The necessity of doing so with members of the *campestre* group of the genus *Phylomonas* has been stressed by Burkholder (6). In the present studies, nitrogen was

TABLE II

ACID FERMENTATION* AT 22° TO 26° C., OF 15 DIFFERENT CARBOHYDRATES IN AN INORGANIC BASAL MEDIUM ONE MONTH AFTER INOCULATION WITH 16 DIFFERENT BACTERIAL ISOLATES IN PURE CULTURE

Accession number of pure cultures	Dextrose	Filt. d-levulose	d-mannose	d-galactose	L-rhamnose	Inositol	Filt. sucrose	Filt. maltose	Filt. lactose	Filt. raffinose	Inulin	Starch	Salicin	d-mannitol	Dulcitol	No carbohydrate
<i>X. translucens</i> f. sp. <i>undulosa</i>																
191	++	+	-	+	-	-	++	-	+	-	-	-	-	-	-	-
385	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
473	++	++	-	+	-	-	++	-	++	-	-	-	+	-	-	-
481	++	+	-	++	-	-	++	-	+	-	-	-	-	-	-	-
618	++	+	-	+	-	-	++	-	+	-	-	-	-	-	-	-
884	++	++	+	++	-	-	++	-	++	-	-	-	-	-	-	-
<i>X. translucens</i> f. sp. <i>hordei-avenae</i>																
239	++	-	++	+	-	-	+	-	-	-	-	-	-	-	-	-
349	+	-	-	+	-	-	++	-	++	-	-	-	+	-	-	-
377	++	+	-	++	-	-	++	-	++	-	-	-	-	-	-	-
451	+	+	+	++	-	-	+	-	++	-	-	-	-	-	-	-
1011	++	+	-	++	-	-	++	-	++	-	-	-	-	-	-	-
1174	++	-	++	++	-	-	++	-	++	-	-	-	-	-	-	-
<i>X. translucens</i> f. sp. <i>cerealis</i>																
1027	++	-	-	+	-	-	++	-	++	-	-	-	-	-	-	-
<i>P. atrofaciens</i>																
909	++	++	++	++	+	++	++	-	-	++	-	-	-	-	++	-
<i>P. coronafaciens</i>																
1014	++	++	++	++	+	++	++	-	-	++	-	-	-	-	++	-
<i>P. medicaginis</i> var. <i>phaseolicola</i>																
1090	++	++	++	++	-	-	++	-	-	++	-	-	-	-	-	-
Control																
None	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* No visible gas production occurred during the fermentation of any of the carbohydrates by any of the organisms.

NOTE: ++ = lowering of pH by 1.0 or more; + = lowering of pH by 0.5 to 0.9; - = no change or lowering of pH by less than 0.5.

used in inorganic form only. All tests were made in duplicate and the results of the two tests averaged. If the resultant mean indicated a lowering in pH amounting to 1.0 or more, the symbol ++ was used; if from 0.5 to 0.9, the symbol +, and, if less than 0.5 or if no change occurred, the symbol - was used. In a few cases it was necessary to repeat the tests owing to lack of growth in one or both the duplicate tubes (Tables II and III).

In general it may be said that the three *formae speciales* of *X. translucens* included in the present studies were capable of digesting dextrose, *d*-levulose, *d*-mannose, *d*-galactose, sucrose, lactose, and salicin; but not *l*-rhamnose, inositol, maltose, raffinose, inulin, starch, mannitol, and dulcitol.

TABLE III

ACID FERMENTATION* AT 22° TO 26° C., OF 15 DIFFERENT CARBOHYDRATES IN AN INORGANIC BASAL MEDIUM TWO MONTHS AFTER INOCULATION WITH 16 DIFFERENT BACTERIAL ISOLATES IN PURE CULTURE

Accession number of pure cultures	Dextrose	Filt. <i>d</i> -levulose	<i>d</i> -mannose	<i>d</i> -galactose	<i>l</i> -rhamnose	Inositol	Filt. sucrose	Filt. maltose	Filt. lactose	Filt. raffinose	Inulin	Starch	Salicin	<i>d</i> -mannitol	Dulcitol	No carbohydrate
<i>X. translucens</i> f. sp. <i>undulosa</i>																
191	++	++	++	++	-	-	++	-	++	-	-	-	-	-	-	-
385	++	++	++	++	-	-	++	-	++	-	-	-	-	-	-	-
473	++	++	++	++	-	-	++	-	++	-	-	-	+	-	-	-
481	++	++	++	++	-	-	++	-	++	-	-	-	++	-	-	-
618	++	++	++	++	-	-	++	-	+	-	-	-	+	-	-	-
884	++	++	++	++	-	-	++	-	++	-	-	-	+	-	-	-
<i>X. translucens</i> f. sp. <i>hordei-avenae</i>																
239	++	++	++	++	-	-	++	-	++	-	-	-	-	-	-	-
349	++	++	++	++	-	-	++	-	++	-	-	-	-	-	-	-
377	++	++	++	++	-	-	++	-	++	-	-	-	+	-	-	-
451	++	++	++	++	-	-	++	-	++	-	-	-	+	-	-	-
1011	++	++	++	++	-	-	++	-	++	-	-	-	+	-	-	-
1174	++	++	++	++	-	-	++	-	++	-	-	-	+	-	-	-
<i>X. translucens</i> f. sp. <i>cerealis</i>																
1027	++	++	++	++	-	-	++	-	++	-	-	-	+	-	-	-
<i>P. atrofaciens</i>																
909	++	++	++	++	++	++	++	+	-	++	-	-	-	++	-	-
<i>P. coronafaciens</i>																
1014	++	++	++	++	++	++	++	-	-	++	-	-	-	++	-	-
<i>P. medicaginis</i> var. <i>phaseolicola</i>																
1090	++	++	++	++	-	-	++	-	-	++	-	-	-	++	-	-
Control																
None	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* No visible gas production occurred during the fermentation of any of the carbohydrates by any of the organisms.

NOTE: ++ = lowering of pH by 1.0 or more; + = lowering of pH by 0.5 to 0.9; - = no change or lowering of pH by less than 0.5.

In the studies made by Dowson (10), on the carbohydrate fermentation of several organisms in an inorganic basal medium, incubation was continued for only one month. The single culture of *X. translucens* obtained by him from Australia produced acid in dextrose, sucrose, and lactose, but not in mannose, maltose, and salicin. The results given in Table II confirm his findings, but those in Table III show that, given sufficient time (two months), some isolates of *X. translucens* are capable of digesting mannose and salicin.

To determine the limits of the diastatic capabilities of the special forms of *X. translucens*, several tests were necessary. As is shown in Tables II and III, all the test and control cultures failed to ferment starch in a mineral basal medium. All the starch tubes gave negative results even after 17 wk. of incubation at 22° to 26° C. Apparently no hydrolysis took place under these conditions, yet, when peptone beef broth was used as a basal medium, definite hydrolysis of the starch was effected in 21 days by all the cultures (Table IV).

TABLE IV

RESULTS OF TESTS FOR THE HYDROLYSIS OF STARCH BY PURE CULTURES OF THREE SPECIAL FORMS OF *X. translucens* IN A MEDIUM CONSISTING OF PEPTONE BEEF BROTH CONTAINING 0.2% SOLUBLE STARCH

Accession number of pure cultures	Reaction of broth after both periods ¹ of incubation	Colour with iodine after acidification ²		Presence of reducing sugars; incubation, 3 wk. ³
		Incubation, 2 wk.	Incubation, 3 wk.	
<i>X. translucens</i> f. sp. <i>undulosa</i>				
191	Alkaline	Almost colourless	Colourless	Trace
385	Alkaline	Purple	Colourless	Trace
473	Alkaline	Colourless	Colourless	Trace
481	Alkaline	Blue	Reddish	Trace
681	Alkaline	Colourless	Colourless	Trace
884	Alkaline	Colourless	Colourless	Trace
<i>X. translucens</i> f. sp. <i>hordei-avenae</i>				
239	Alkaline	Colourless	Colourless	Slight
349	Alkaline	Blue	Colourless	Trace
377	Alkaline	Colourless	Colourless	Trace
451	Alkaline	Colourless	Colourless	Trace
1011	Alkaline	Colourless	Colourless	Trace
1174	Alkaline	Colourless	Colourless	Slight
<i>X. translucens</i> f. sp. <i>cerealis</i>				
1027	Alkaline	Purple	Colourless	Trace
Control				
Uninoculated	Neutral	Blue	Blue	None

¹ 5 drops phenol red added to 1 cc. of test broth.

² 1 cc. test broth + 1 drop N/1 hydrochloric acid + 1 cc. saturated aqueous solution of iodine.

³ Equal volumes of test broth and Benedict's reagent mixed, heated in boiling water-bath for five minutes and cooled. Trace denotes a greenish tinge. Slight denotes a definite green colour.

Similar results were obtained with 23 additional cultures. These results indicate that *X. translucens* is capable of digesting starch, but only in the presence of a basal medium that in itself permits good growth. As all the cultures developed abundant growth in peptone-beef-starch broth by the third day after inoculation without causing any hydrolysis until several days later, *X. translucens* may be considered to be weak in diastatic enzymes.

SEROLOGICAL STUDIES

The difficulties encountered in distinguishing between bacterial black chaff and other diseases having somewhat similar symptoms, led Belenkii and Popova (3) to propose a serological method of determining the presence of the bacterial black chaff organism. Gorlenko, Naidenko, and Klykov (17) believed this method could be adapted to the detection of bacterial black chaff in seed wheat. According to their tests, the bacterial black chaff organism could be determined definitely even when grown in association with other organisms.

To test the specificity of serological determination in relation to the cultures included in the present study, a serum was prepared that was capable of agglutinating *X. translucens* f. sp. *undulosa*. This serum caused complete agglutination of all cultures of *X. translucens* tested with it by the macroscopic agglutination method described above, which is, essentially, that of Conn *et al.* (9). The two cultures of f. sp. *cerealis* became agglutinated at higher serum dilutions than did any culture of f. sp. *undulosa* or f. sp. *hordei-avenae* (Table V). In one culture, No. 239, of f. sp. *hordei-avenae*, the clumping was more finely divided than in any other culture, but the relation of agglutination to serum dilution was the same with it as with most other cultures of the species. Complete agglutination occurred with two of the test cultures in all final serum dilutions of 1/20 to 1/320, inclusive, with 11 cultures in all dilutions from 1/20 to 1/160, and with one culture in only the dilutions from 1/20 to 1/80. Although both *P. atrofaciens* and *P. medicaginis* var. *phaseolicola* failed to show any agglutination, *P. coronafaciens* became completely agglutinated in a final dilution of 1/20. In this connection it may be mentioned that the serum was obtained from a rabbit fed partly on oats, although wheat was avoided. There is thus a possibility that the serological activity toward *P. coronafaciens* may have been acquired by ingestion of that organism along with the oats.

Discussion

For many years the classification of bacterial pathogens has been in a state of flux. That many different generic names are in use for organisms of the same description is too well known to require discussion (5, 7). But now, with the recognition of at least three groups present in Bergey's genus *Phytophthora*, and the naming of one of these groups *Xanthomonas* by Dowson (10, 11), there appears to be no further grounds for confusion in assigning to the proper genus plant pathogenic bacteria that fall within the limits of Dowson's genus.

TABLE V

MACROSCOPIC AGGLUTINATION REACTIONS AT 50° C. OF SEVERAL PURE CULTURES OF BACTERIAL PATHOGENS WITH SERUM A AT NINE DIFFERENT DILUTIONS

Final dilution	<i>Xanthomonas translucens</i>														<i>P. atro-faciens</i>	<i>P. coronafaciens</i>	<i>P. medicaginis</i> var. <i>phaseolicola</i>	Control (saline)	
	<i>f. sp. undulosa</i>						<i>f. sp. hordei-aeneae</i>												<i>f. sp. cerealis</i>
	Accession number of cultures																		
	191	385	473	481	618	884	239	349	377	451	1011	1174	1027	1236 ³					
1-20	C	C	C	C	C	C	C ¹	C	C	C	C	C	C	C	N	C	N	N	
1-40	C	C	C	C	C	C	C ¹	C	C	C	C	C	C	C	N	++	N	N	
1-80	C	C	C	C	C	C	C ¹	C	C	C	C	C	C	C	N	Trace	N	N	
1-160	C	++	C	C	C	C	C ¹	C	C	C	C	C	C	C	N	N	N	N	
1-320	++	+	+	++	+	+	++ ¹	++	++	++	++	++	C	C	N	N	N	N	
1-640	N	N	N	+	+	N	+	+	++	+	+	+	++	++	N	N	N	N	
1-1280	N	N	N	N	N	N	N	N	N	N	N	N	++	+	N	N	N	N	
1-2560	N	N	N	N	N	N	N	N	N	N	N	N	+	Trace	N	N	N	N	
1-5120	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
Saline	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	

¹ Clumps of finer texture than with other agglutinating cultures.

² Isolated from wheat heads collected at Austin, Man., in 1940.

NOTE: C = complete agglutination; ++, +, and trace = degrees of partial agglutination; N = no agglutination.

As his genus has priority according to the International Rules of Botanical Nomenclature, it is used in the present paper.

In the species description, as emended to embrace the five closely-related organisms, only those characters essential to the determination of the species have been included. They are all characters that have come to be recognized as determinative in the group of species with which *Xanthomonas translucens* is associated taxonomically. Characters that are not included in the species description are regarded as non-determinative characters. Several of the non-determinative characters in this species admittedly may be considered of determinative value in other species. For example, all the test cultures were Gram-negative, but this character was not included in the determinative description.

During recent years it has become increasingly apparent that the *formae speciales* of Eriksson (12), and their subdivisions, are extremely useful in the classification of the rusts and other pathogenic fungi. The utility of these groupings in the study of pathogenic bacteria is beginning to be realized now. The group species *Xanthomonas translucens* exhibits what is perhaps the best example of physiological specialization in the bacterial phytopathogens and it seems significant that the same host plants are encountered with it as in the cereal rusts. In fact, a close parallel can be drawn between the physiological specialization in *Xanthomonas translucens* and in *Puccinia graminis* Pers.; in the latter the group concept has been recognized for nearly half a century. In both groups several *formae speciales* occur in cereals and in both groups the *formae speciales* differ in their ability to attack seedlings of wheat, oats, barley, and rye. *X. translucens* f. sp. *undulosa* is an analogue of *P. graminis* f. sp. *Tritici* Erikss. and Henn., since both of them occur chiefly on wheat, but can also attack barley and rye. Neither attacks oats in nature, yet both will infect oats slightly following the inoculation of meristematic tissues. Similarly *X. translucens* f. sp. *cerealis* is an analogue of the hybrid produced by Johnson and Newton (20) from the cross *P. graminis* f. sp. *Tritici* × *P. graminis* f. sp. *Avenae* Erikss. and Henn. Both the bacterium and the hybrid rust infect seedlings of wheat, oats, barley, and rye. *X. translucens* f. sp. *secalis* resembles somewhat in pathogenic capabilities *P. graminis* f. sp. *secalis* Erikss. and Henn., but as far as is known *X. translucens* f. sp. *hordei* and f. sp. *hordei-avenae* have no counterparts in *P. graminis*. The *P. graminis* *Hordei* of Freeman and Johnson (14) was capable of attacking wheat and rye in addition to barley and so would clearly not be analogous to either of them, as neither of them attacks wheat and rye.

The means by which the special forms have arisen in *X. translucens* is still quite unknown. Consequently the comparison between specialization in it and in *P. graminis* cannot be extended at present to include the method of origin of the special forms. That the mechanism of variation is fairly well understood in *P. graminis*, but not in *X. translucens*, does not alter the fact that variations definable in terms of pathogenic capabilities exist in both of these species. Acceptance of the concept of special forms in *X. translucens*

would seem to be adequately justified by the fact that pure cultures of the various special forms retain their identity during successive passages through the host plants and in successive transfers on artificial media.

The non-determinative studies reported above have demonstrated a very close similarity between the different special forms of *X. translucens*. In some cultures, differences in rate of acid production were observed, but these differences were as great between different isolates of the same special form as between different special forms. No correlation could be found between any cultural character and pathogenicity.

Differences in pathogenic capabilities occur between different isolates of the same *forma specialis* of *X. translucens*. For example, in inoculation studies with *X. translucens* f. sp. *undulosa*, isolate No. 884 consistently caused a weaker reaction than isolate No. 385 on seedlings of Thatcher wheat. Such differences in pathogenicity suggest that the need may yet arise of recognizing subordinate groups (e.g., races) within the special forms, as has long been found necessary in the rusts.

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TWO NEW CANADIAN SMUTS¹

BY IVAN H. CROWELL²

Abstract

Burrillia anomala Crowell forms irregularly elongated spots on the leaves of *Sparganium diversifolium* Graebner var. *acaule* (Beeby) Fernald & Eames. The spore-ball of this smut is composed of readily disjoined spores, an occurrence reported in only one other species of the genus, namely, *B. acori*. Both of these species are recorded only from Ontario. *Entyloma peninsulae* Crowell forms linear sori on leaves of *Zizania aquatica* L. It differs from *E. lineatum* (Cooke) Davis in having much longer sori and considerably smaller spores. *E. peninsulae* is recorded only from the type locality in New Glasgow, N.S.

1. *Burrillia anomala* Crowell, sp. nov. (Figs. 1, 2, 3, 4).

Soris in foliis, ellipticis vel irregularibus, brunneis; sporarum glomerulis in lacunis, solitariis vel pluribus, subglobosis, flavescentibus vel brunneis, circa 200–300 μ crassis; sporis leve, hyalinis vel pallide brunneis, subglobosis vel ellipsoideis, 11–9 \times 10–8 μ , plus minusve 9.8 \times 9.0 μ tunica tenui; conidiis non visis.

On *Sparganium diversifolium* Graebner var. *acaule* (Beeby) Fernald & Eames, Denton's Bay, Bear Island, Lake Timagami, Ont., Sept. 12, 1929, H. S. Jackson, H. H. Whetzel, and Geo. E. Thompson; type.

Type deposited in the Macdonald College Mycological Herbarium, No. 948.

Other specimen examined: On the same host, collected near the type locality Aug. 16, 1930, I. L. Connors, No. 1595, Mycological Herbarium of the Division of Botany and Plant Pathology, Department of Agriculture, Ottawa, Ont.

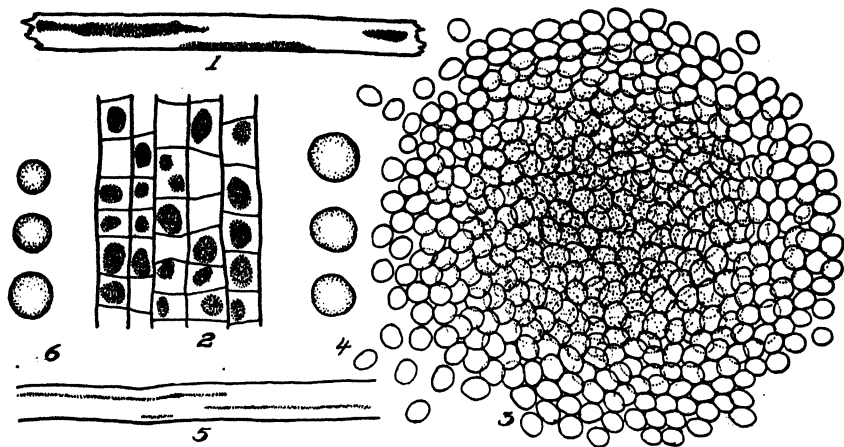
In his description of the genus *Burrillia*, Setchell (4) states "Sorus (spore-ball) compact, not separating into its elements on being crushed . . ." This condition seems to be true for all but two species of the genus, namely, *B. acori* Dearness (3) and *B. anomala*, both of which are reported only from Ontario. Spore-balls of these species are unusual in that the spores are very loosely held together. Dry spore-balls may be described as friable, since the spores are readily disjoined, becoming powdery, under slight pressure. In a microscopic mount many spores will float free in the mounting medium.

A major distinction between the genera *Burrillia* and *Entyloma* is that spores of species of *Entyloma* are formed singly or free from one another, though they may adhere loosely in the sorus, while spores of species of *Burrillia* tend to be firmly agglutinated into spore-balls of rather definite size and shape. An intermediate condition is represented by *B. acori* and *B. anomala* in which the spores are more or less powdery when mature, certainly not agglutinated, and yet are formed in clusters of definite size and shape. Thus one is left somewhat in doubt as to whether these species may be more correctly assigned to the genus *Entyloma* with its more or less free spores or to the genus

¹ Manuscript received in original form, November 25, 1941, and as revised, March 6, 1942.

Contribution from the Department of Plant Pathology, Macdonald College, Macdonald College P.O., Que. Macdonald College Journal Series No. 157.

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FIGS. 1 TO 4. *Burrillia anomala*. FIGS. 5 AND 6. *Entyloma peninsulæ*.

FIG. 1. Habit sketch of lesions on leaf. $\times 1$ approx. FIG. 2. Spore-balls in leaf tissue. $\times 20$ approx. FIG. 3. Diagrammatic sketch of a partially disjoined spore-ball. $\times 375$ approx. FIG. 4. Spores. $\times 750$ approx. FIG. 5. Habit sketch of linear lesions on leaf. $\times 1$ approx. FIG. 6. Spores. $\times 750$ approx.

Burrillia with its agglutinated spore-balls. The author has preferred to accept the interpretation of Dearness (3) and place the new species in the genus *Burrillia*.

2. *Entyloma peninsulæ* Crowell, sp. nov. (Figs. 5 and 6).

Sori in foliorum lacunis, linearibus, brunneis; sporis ovoideis vel subglobosis, levè, hyalinis vel pallidissime brunneis, tunica tenui, $8-5 \times 7-5 \mu$, plus minusve $6.2 \times 5.6 \mu$; conidia non visis.

On *Zizania aquatica* L., New Glasgow, N.S., Aug. 20, 1906, W. P. Fraser; type.

Type deposited in the Macdonald College Mycological Herbarium, No. 783.

Entyloma peninsulæ occurs on the same host as *E. lineatum* (Cooke) Davis (2). It is rather unusual to find two species of *Entyloma* on one host species. Yet the sori of *E. peninsulæ* are so much longer (up to 10 cm. as compared with 0.5 to 3 mm. for those of *E. lineatum*) and its spores so much smaller (8.5×10.6 as compared with $5.6 \times 6.2 \mu$ for *E. lineatum** (1, p. 60) that it seems justifiable to consider these fungi as representing distinct species.

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* *Fungi Columbiana* No. 3224.

Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 20, SEC. C.

JUNE, 1942

NUMBER 6

THREE STRAINS OF CUCUMBER MOSAIC OCCURRING ON TOBACCO IN ONTARIO AND QUEBEC¹

By J. H. H. PHILLIPS²

Abstract

Cucumber mosaic was found affecting tobacco plantings in both Ontario and Quebec. From diseased material collected in these regions three strains were isolated and designated as Strains 1, 2, and 3. Strain 1 most closely resembled typical cucumber mosaic in its symptoms on tobacco and tomato. Strain 3 produced a similar type of mottle to Strain 1, but was generally more severe and consistently produced severe leaf narrowing on tomato. Strain 2 was easily recognized by its ability to produce necrotic rings on the inoculated leaves of burley tobacco varieties and the tendency of affected plants to recover from the initial symptoms. The three strains retained their identity through a large number of serial inoculations.

Investigations demonstrated that a severe type of streak was produced when tomato plants were inoculated with a combination of cucumber mosaic virus (Strain 3) and potato X virus.

Unlike tobacco mosaic virus, the virus of cucumber mosaic was unable to survive over winter in plant tissue in the soil. Field observations indicated that dissemination of cucumber mosaic in tobacco plantings was effected by insect vectors.

Since Johnson (6) first described cucumber mosaic on tobacco, it has been recognized as being widely distributed on this host. Valteau and Johnson (10) in 1928 reported it from Kentucky as "Puffed" disease, and Johnson (5) in 1930 described three strains occurring on tobacco in Kentucky. Several strains have since been described by various workers and the symptoms and properties of the virus have been extensively studied.

In 1939, considerable cucumber mosaic was observed at St. Catharines in tobacco plots in which tobacco mosaic was being studied. At that time the symptoms, especially in the early stages of infection, were easily confused with those of tobacco mosaic and the identity of the virus concerned was often ascertained only by inoculation to other hosts. There was also considerable variation in the symptoms produced, which suggested the possibility of more than one strain of cucumber mosaic being involved. For this reason a study of the symptoms of cucumber mosaic as it occurs in Ontario was considered advisable.

¹ Manuscript received February 12, 1942.

Contribution No. 694 from the Division of Botany, Science Service, Department of Agriculture, Ottawa, Canada.

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Distribution and Importance

Tobacco leaves showing symptoms of cucumber mosaic have been received at St. Catharines from the tobacco growing areas of both Ontario and Quebec. Though cucumber mosaic occurs in both the old and new tobacco belts in Ontario, it does not appear to be widespread or to cause much damage to the crop. In Quebec, however, where tobacco is grown in comparatively small plots often adjacent to vegetable crops such as potatoes, cucumbers, tomatoes, etc., cucumber mosaic on tobacco is more prevalent.

Investigations at St. Catharines indicate that certain strains of the cucumber mosaic virus are capable of causing severe injury to tobacco, while other strains cause little or no injury beyond mild stunting. Because of its superficial resemblance to tobacco mosaic, many growers in Ontario and Quebec are not aware of the occurrence of this disease on tobacco.

Material and Methods

Tobacco and weed hosts infected with cucumber mosaic were collected from both Ontario and Quebec. Of the many strains 'isolated' from this material, three gave consistent differences on a certain host range and were used for further study. Some 2000 plants including 18 varieties of tobacco and 16 other hosts were inoculated during the course of these investigations.

Inoculations were made by the leaf rubbing method. Infected tissue was macerated in a sterile mortar, diluted with one part of tap water and gently rubbed over the leaf of the healthy plant with a pad of sterile gauze. Juice for thermal inactivation tests was prepared in a similar manner and then centrifuged for two to three minutes. It was then poured into 3 cc. stoppered glass tubes and plunged into a constant temperature water-bath for the desired period.

Plants were grown in 5-in. pots and usually were inoculated as soon as two or three large leaves had developed. They were kept under daily observation and symptoms were recorded as they appeared. All plants were grown in screened greenhouse compartments which were fumigated regularly to control insects. With one or two exceptions all experiments were repeated several times and doubtful transmissions of the virus were checked by inoculations back to tobacco.

Symptoms of the Three Strains

Under greenhouse conditions, the symptoms produced by the three strains on cucumber var. Chicago Pickling were very similar and resembled those described for cucumber mosaic by Doolittle (3) except that no mottling was observed on the fruit. When small cucumber plants were inoculated, they usually died before setting fruit. Any fruits that were produced were small and misshapen with undeveloped narrowed tips. Plants inoculated in the field showed similar symptoms, though the plants were not killed, a small rosette of leaves remaining alive at the tips of the vines.

The three strains were differentiated, however, by the symptoms they produced on tobacco and tomato, as shown in Table I.

TABLE I
DIFFERENTIATION OF CUCUMBER MOSAIC STRAINS ON TOBACCO AND TOMATO

Host	Strain 1	Strain 2	Strain 3
Tobacco var. Harrow Velvet	(i) Large, diffused primary pale green lesions (ii) Vein clearing and paling of young leaves (iii) Blotchy pale green mottle, leaf tips narrowed and chlorotic, some stunting of plants	(i) Large primary yellow lesions with concentric necrotic rings (Fig. 1) (ii) Vein clearing and general paling or vein-banding of young leaves with fine necrotic spotting (iii) Fine mosaic mottle often with definite ring spotting, plants tend to recover from the initial symptoms but are paler than normal	(i) Large, diffused primary yellow lesions, may become necrotic (ii) Vein clearing and paling of young leaves (iii) Blotchy yellow mottle, leaves narrowed with elongated, chlorotic tips, some leaf distortion and blistering, severe stunting
Tomato var. Grand Rapids	(i) Primary pale green lesions (ii) Vein clearing and paling of young leaves (iii) Coarse blotchy pale green mottle, leaf narrowing usually slight, some stunting	(i) Necrotic local lesions (ii) Vein clearing of young leaves (iii) Plants may be paler than normal, no mottle	(i) Large diffuse primary yellow lesions (ii) Paling or yellowing of young leaves (iii) Severe leaf narrowing, 'fern leaf'. Plants partially recover from severe leaf narrowing, have yellow mottle, severe stunting

All of the following hosts were also successfully inoculated with each of the three strains:—*Nicotiana glutinosa* L., *N. rustica* L., *N. Langsdorffii* Wunn., *N. sylvestris* Spegaz & Comes., *N. glauca* Grah., *N. Bigelovii* S. Wats., *Physalis angulata* L., *Capsicum frutescens* L., *Nicandra physalodes* (L.) Pers., *Phytolacca decandra* L., *Antirrhinum* sp., *Datura Stramonium* L., *Zinnia* sp., and *Petunia* sp.

Strains 1 and 3 generally produced a coarse, blotchy mottle of a similar type on all of these hosts with or without primary yellow lesions. Strain 3, however, could usually be distinguished by its more contrasting mottle and a tendency to cause severe stunting. On *Datura Stramonium* only a mild ring spot type of mottle was produced by either of these two strains. This tended to fade and affected plants later appeared almost normal. Strain 2 consistently produced a mild mottle of a finer type than Strains 1 and 3 without the leaf narrowing or severe stunting. On the following hosts this mottle was accompanied by a fine necrotic or ring spotting similar to that produced on tobacco:—*N. rustica*, *N. glutinosa*, *P. angulata*, *N. physalodes*. On all the

other hosts a systemic mild mottle only was produced and plants often tended to recover and produce normal growth.

Under conditions of poor light and poor plant growth, transmission of cucumber mosaic to tobacco by mechanical injury was often difficult. Symptoms were slow in appearing and showed considerable variation in severity. Primary yellow lesions were often not visible until after systemic symptoms had appeared, and inoculated leaves tended to become necrotic. Under winter conditions the symptoms on tomato produced by Strain 1 may approach the 'fern leaf' symptoms of Strain 3, but under more favourable growing conditions Strain 1 produced only a coarse mottle, whereas Strain 3 produced the severe leaf narrowing symptoms. Attempts to duplicate these symptoms in the field failed, since considerable difficulty was experienced in inoculating tomato plants and there was a high incidence of natural infection by both cucumber mosaic and tomato mosaic viruses.

Under field conditions, the symptoms of cucumber mosaic on tobacco corresponded to those produced by the three strains in the greenhouse. Strain 2 could be distinguished readily from Strains 1 and 3 by the large, distinct necrotic rings on the inoculated leaves, the necrotic flecking and ring spotting on the leaves which showed the primary systemic symptoms and the tendency of the plants to recover from the mottle. Though Strains 3 and 1 produced a similar type of coarse mottling, Strain 3 induced a more severe type of breakdown on inoculated leaves and caused more pronounced narrowing of leaves and more severe stunting of plants. The three strains all tended to produce a necrotic breakdown of the older leaves, especially along the veins, in the form of elongated necrotic spots, line patterns, and ring spots. It was interesting to note that in the field burley varieties developed more severe symptoms and a more contrasting mottle than flue-cured varieties. This was especially noticeable with Strain 3 which produced a distinct yellow mottle on the burley varieties and a much less contrasting pale green mottle on the flue-cured varieties.

In comparison with tobacco mosaic the symptoms of cucumber mosaic on tobacco, though usually less severe, were nevertheless more chlorotic. Unlike tobacco mosaic the young leaves at the top of a plant infected with cucumber mosaic, after the initial systemic symptoms, appeared almost normal with only scattered pale green or yellow areas or a mild mottle. As these leaves expanded they developed the characteristic blotchy mottle or general paling characteristic of the strain. In the early stages of infection on tobacco it was often difficult to distinguish cucumber mosaic from tobacco mosaic in that they both produced a vein clearing, and often a pale green to yellow vein banding of the young leaves. In the later stages of infection, however, plants infected with tobacco mosaic virus showed a uniform mottling of the leaves with blistering and distortion, whereas those infected with cucumber mosaic virus showed a coarse, blotchy mottle with only occasional small blisters, but with the leaf tips chlorotic and narrowed.



FIG. 1. *Strain 2 showing the necrotic rings on the inoculated leaves and the paling and necrotic flecking of young leaves of Harrow Velvet.*

Cucumber Mosaic in Relation to Tomato Streak

During the course of the investigation cucumber mosaic was also recovered from greenhouse tomato plants showing typical streak symptoms. The possibility that cucumber mosaic may be involved in the production of a 'streak' of tomatoes was investigated.

Tomato plants in the greenhouse were inoculated with one of the three strains of cucumber mosaic, together with one of the following: (a) *Nicotiana* Virus 1 (9)—ordinary tobacco mosaic which produced primary yellow lesions and a systemic mosaic mottle on tomato; (b) *Lycopersicum* Virus 1—a mild strain of tomato streak virus which produced necrotic local lesions on tobacco var. Yellow Mammoth but only a systemic mosaic mottle on tomato; (c) *Solanum* Virus 1 (potato X virus)—a strain recovered from potato, which produced a rather severe mottle on tomato with some necrotic ring spotting as the initial symptoms, but from which plants tended to recover.

It was found that Strain 1 was almost entirely masked by each of the three viruses with which it was inoculated. Strain 2 tended to produce necrotic local lesions when inoculated along with either tomato streak virus or potato X virus and increased the amount of necrotic spotting in a potato X virus combination. Strain 3 inoculated along with either tobacco mosaic virus or tomato streak virus produced a necrosis on tomato of a relatively mild type as compared to the usual 'combination streak' encountered. With potato X virus, however, Strain 3 produced streak symptoms of a severe type closely resembling those produced by potato X virus plus tobacco mosaic virus.

Properties

All three strains failed to survive heating to 65° C. for 10 min. and failed to withstand drying in the leaf tissue. Great difficulty was experienced in recovering the virus from leaves dried for more than 48 hr. at room temperature.

Overwintering

Doolittle and Walker (4) have shown that the virus of cucumber mosaic overwinters in perennial hosts or in the seed of wild cucumber and does not persist in the soil for any length of time. On the other hand Johnson (7) in Wisconsin and Berkeley (2) in Ontario have shown that the virus of tobacco mosaic is capable of wintering over in the soil and infecting tobacco, if tobacco follows tobacco in the rotation. However, since cucumber mosaic is often present in tobacco plantations in Ontario, it was considered advisable to test for the possibility of its overwintering in the soil under Ontario conditions.

Accordingly, in the spring of 1940 all plants in a plot of 200 tobacco plants (plot No. 1) were inoculated with cucumber mosaic and in October of the same year the inoculated plants were cut up and incorporated with the soil. A similar plot was inoculated with tobacco mosaic virus (plot No. 2) and treated in a similar manner. These plots were cultivated only when the plants were very small and every care was taken to avoid contamination from outside

sources. A check plot of 200 uninoculated plants (plot No. 3) treated similarly remained free from either cucumber or tobacco mosaic throughout the season. At intervals throughout the winter samples of soil from these plots were brought into the greenhouse and tested for the presence of virus by rubbing gently the leaves of *N. glutinosa* with a soil suspension of these samples which had been previously stirred in a sterile mortar, in order to extract any virus present in the soil. The remaining soil was put into 5-in. pots and planted with young, vigorously growing tobacco plants. The results of these tests are shown in Table II.

TABLE II

THE RESULTS OF TESTS MADE DURING THE WINTER WITH SOIL FROM PLOTS IN WHICH CUCUMBER MOSAIC AND TOBACCO MOSAIC PLANTS HAD BEEN INCORPORATED WITH THE SOIL

Date of taking sample	Soil from tobacco mosaic plot No. 2		Soil from cucumber mosaic plot No. 1	
	Soil suspension inoculation	Plants potted in soil	Soil suspension inoculation	Plants potted in soil
Dec. 3, 1940	3/3*	4/3	3/0	4/0
Jan. 3, 1941	4/4	4/2	4/0	4/0
Feb. 1, 1941	4/4	4/2	4/0	4/0
Mar. 31, 1941	4/4	4/0	4/0	—
May 17, 1941	4/3	4/0	—	—

* Upper figure indicates number of plants inoculated or planted, lower figure indicates number of plants infected one month from the date of inoculation.

The results recorded in Table II would suggest that the cucumber mosaic virus did not persist in the soil during the winter of 1940, whereas the tobacco mosaic virus did overwinter in the soil.

In the spring of 1941 when these plots were replanted to tobacco, cucumber mosaic appeared within one month of planting, but it was not significantly more prevalent in plot No. 1 than in plot No. 2 and in No. 3. On the other hand, plot No. 2 showed 4% infection with tobacco mosaic one month after replanting, but only one plant infected with tobacco mosaic appeared in plot No. 1, thus indicating that tobacco mosaic had overwintered in the soil. The uniformity of infection with cucumber mosaic in both the inoculated and non-inoculated plots in the spring of 1941 would further indicate that in plot No. 1 the soil was not the source of infection for these plants. It is more probable that perennial weeds adjacent to the plots served to carry over the virus.

In a series of plots for the study of other viruses it was observed that cucumber mosaic spreads much more rapidly than tobacco mosaic and that the location of infected plants could not be correlated with the soil or cultural practices. Rather the observations suggested that infection was more likely to be due to insect vectors, especially since the aphid, *Macrosiphum gei* Koch, occurs quite commonly on tobacco at St. Catharines and in limited tests readily transmitted cucumber mosaic to this host.

Discussion

The results of these investigations of cucumber mosaic on tobacco in Ontario are for the most part in agreement with those of workers in other parts of the world. Though only three distinct strains were encountered in Ontario and Quebec, more might have been obtained if the selective method used by Price (8) in his separation of yellow strains had been employed. The strains recorded here were selected because they were of common natural occurrence. They gave a consistent symptom picture throughout a large number of transfers and were quite distinct and identifiable in the field in comparable infections. The identification of these strains with those described by previous workers presents difficulties but in general Strain 1 resembles the cucumber mosaic of Doolittle (3) in America and the yellow mottle mosaic of cucumber of Ainsworth (1) in England.

Strain 3 appears to be similar in its action on tomato to the Strain 3 used by Valteau and Johnson (11) in their experiments with 'streak' of tomato. Strain 2 may be a new strain, as it could not be identified with any strain encountered in the literature. It is not suggested that these are pure strains, as they may be relatively stable combinations of more than one strain. Their reaction on cucumber, their thermal death point and host range do, however, tend to confirm that they are all strains of *Cucumis Virus 1* (9).

The apparently limited occurrence of cucumber mosaic in tobacco plantings in Ontario may be due to it being confused with tobacco mosaic. At certain stages of infection the symptoms may be very similar but when the typical mottling is present, they are easily distinguished.

Unlike tobacco mosaic which persists over winter in the soil and is spread mainly by contact, cucumber mosaic apparently is spread by insects from overwintering perennial hosts. It is therefore apparent that control measures as applied to tobacco mosaic would not be effective in controlling outbreaks of cucumber mosaic.

Acknowledgment

The author wishes to express his appreciation to Dr. G. H. Berkeley for suggesting and directing this investigation and for his guidance in the preparation of this paper.

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THE BEHAVIOUR OF RESAZURIN IN MILK¹

By C. K. JOHNS²

Abstract

The view that resazurin is not a satisfactory substitute for methylene blue in assessing the hygienic quality of milk has been examined.

Resazurin is equally as sensitive as methylene blue to the metabolic activities of bacteria in milk. It is decidedly more sensitive to the presence of non-bacterial factors in abnormal milks (mastitis, late lactation, etc.) and therefore furnishes a more comprehensive index of the true quality of the milk.

Potentiometric studies with a wide variety of milks have failed to support the view that resazurin exerts such a strong poisoning action in market milks as to complicate the test or interfere with the interpretation of results. No evidence was obtained in support of the claim that differences in poisoning properties of different milks are of sufficient magnitude to affect the results of the test.

The suitability of the dye resazurin for determining the hygienic quality of milk has been questioned seriously by Thornton, McClure, and Sandin (29). These workers state that "a significant proportion of results cannot be interpreted with the reasonable certainty necessary to justify the widespread use of these (resazurin) tests by the dairy industry". Since this view runs counter to those of various other investigators (2, 3, 4, 15, 18, 19, 20, 21, 22, 23), it seemed desirable to examine in some detail the objections raised by the aforementioned workers.

In the paper referred to, two points in particular have been stressed. The first is that the degree of colour change in resazurin-milk mixture does not always correlate satisfactorily with the bacterial content as indicated by the methylene blue reduction test or other method. This springs from the fact, reported by various workers (2, 7, 16, 18, 19, 22, 23, 24, 26, 27), that resazurin is much more sensitive to the presence of non-bacterial factors (leucocytes or substances accompanying them) than is methylene blue. Consequently, milk from diseased or otherwise abnormal udders will show a significant degree of colour change in resazurin during the first few hours of incubation, even though the bacterial content is low. Resazurin is therefore much more useful than methylene blue in detecting such abnormal milks, while equally as valuable in reflecting excessive bacterial numbers.

The influence of these non-bacterial factors, which appear to be responsible for discrepancies between the results of the resazurin "one hour" (23) or "triple reading" (18) tests and the methylene blue reduction test, has been generally recognized by other workers. Thornton *et al.* appear to ignore this influence, preferring to judge the test exclusively on its ability to indicate bacterial numbers. The desirability of a broader concept of milk quality has previously

¹ Manuscript received February 8, 1942.

² Contribution No. 146 (Journal Series) from the Division of Bacteriology and Dairy Research, Science Service, Department of Agriculture, Ottawa.
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been discussed (16, 18). It may also be pointed out that these non-bacterial factors have little influence upon the time required for resazurin to reach the full pink stage (colour No. 16) in market milks (18), as indicated by the close correlation between reduction times of resazurin to pink and methylene blue to white (3, 15, 17). Consequently, if the hygienic quality of milk is to be judged solely by the bacterial content, the resazurin "pink" test may be substituted for the methylene blue test with an appreciable shortening of the reduction time (3, 15).

The second point stressed by Thornton *et al.* is the strong poisoning action of resazurin in milk. Such statements as "milk was immediately overpoised by the resazurin", "the possibilities of overpoising effects", etc., appear throughout their paper. Poisoning action refers to resistance to change in potential upon the addition of an oxidizing or reducing agent. Two factors are concerned; (a) the intensity, represented by the E_o value which characterizes a reversible oxidation-reduction system, and (b) the capacity or concentration factor. Neither the E_o values nor the concentrations of the systems responsible for the initial potential level of milk are known. Ramsdell *et al.* (23) stated that resazurin is slightly more electropositive than methylene blue but gives no E_o value; Thornton *et al.* reported obtaining a perfect two-step titration curve at approximately pH 1, but failed to present their curve or the E_o value for resazurin. Almost the only known factor in a resazurin-milk mixture is the concentration of the dye. Assuming the dye to be 100% pure¹, the customary strength in milk (1 in 200,000 parts) represents a concentration of 0.000022 *M*. It seems most unlikely that this low concentration of an irreversibly² reducing dye system could immediately overcome the initial potential of the milk and force it to a significantly lower level. (This is presumably what Thornton *et al.* mean by the term "overpoising".) The previously published data on the influence of various concentrations of resazurin upon the time-potential curve show that even double the usual concentration of the dye fails to change the potential level of the milk until bacterial activity enters into the picture (17). The only evidence of any poisoning action by the dye manifests itself when the resazurin has been almost completely reduced to the pink component, resorufin. Since the reduction of resorufin to the colourless compound is reversible, poisoning action at this stage is not unexpected. The shorter time required to reach the pink stage, compared with the decolorization of methylene blue, was reported (17) as being mainly attributable to the change in shape of the time-potential curve owing to the poisoning action in the vicinity of the pink stage.

The view of Thornton *et al.* that resazurin is of dubious value because it tends to force the potential of milk downward to a lower level appears to be based mainly upon the few time-potential curves presented in their paper.

¹ By the titanous chloride method, the lot of resazurin used here was found to be 95% pure (Eastman Kodak Company, April 12, 1939).

² While the change from pink to white is reversible, the previous change from initial blue (resazurin) to pink (resorufin) is irreversible in milk (23, 29).

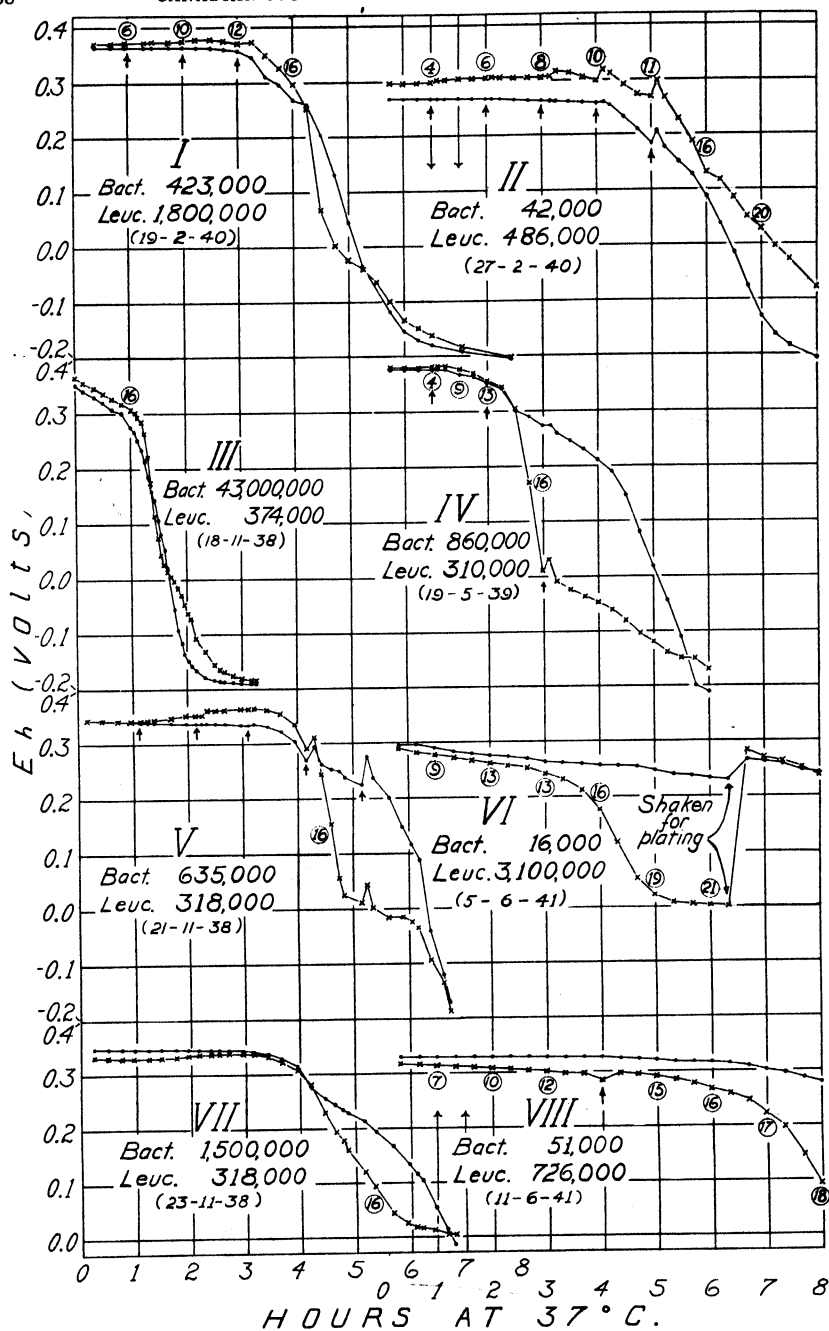


FIG. 1. Time-potential curves of milks with and without resazurin. Nos. I and II = herd milks, Nos. III, IV, V, and VII = vat milks, Nos. VI and VIII = milks from individual cows. Counts made by direct microscopic method, counting 60 fields. Arrows indicate inversion of tubes to redistribute cream layer. Ringed numbers represent resazurin colours (0 = initial, 16 = pink, 24 = discolored). x—x = resazurin; •—• = no dye.

With the exception of Milk 2 in their Fig. 1, these curves differ significantly from those previously published (17). A further series of representative curves is shown in Fig. 1. It will be noted that even with Milks VI and VIII from cows with mastitis, the curve for milk + resazurin closely parallels that for plain milk for several hours. In no instance is there any suggestion that resazurin causes downward displacement of the potential level of the milk during the first few hours. Even in Milk III with an extremely high bacterial content and short reduction time, there is no hint of the "overpoising effect" mentioned by Thornton *et al.*

Two possible explanations for these differences in findings suggest themselves: (1) some inherent difference in the behaviour of their milks and of ours; and (2) differences in the methods employed in measuring potentials.

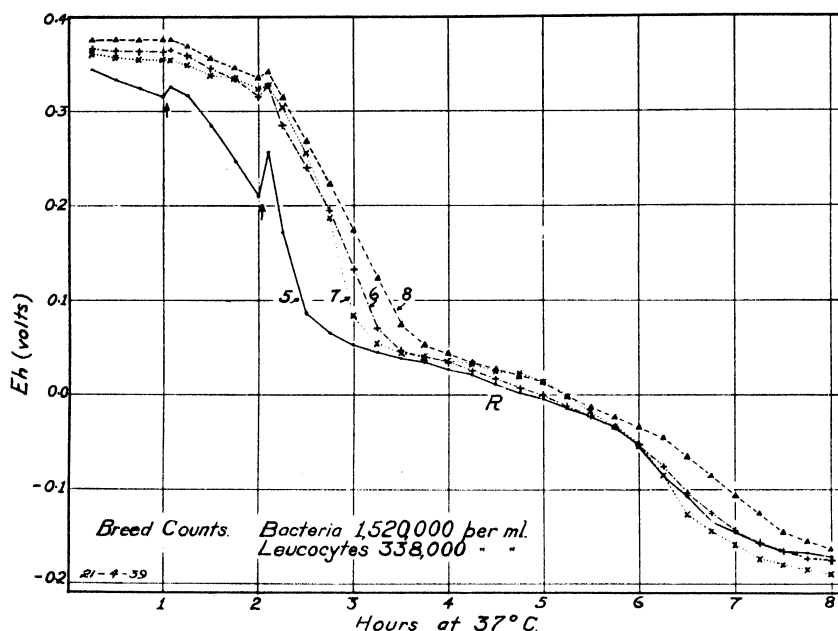


FIG. 2. Time-potential curves for four electrodes in milk + methylene blue. Tubes inverted at points marked \uparrow . Dye reduced at Point R.

We know of no evidence that milks in the Edmonton district behave so differently from those in the Ottawa area that this would account for the above-mentioned differences in the shapes of time-potential curves. On the other hand, it is generally recognized that a single electrode may give aberrant readings upon occasion, even though at other times it behaves normally. The curve for electrode No. 5 in Fig. 2 serves to illustrate this. In this instance the milk contained methylene blue thiocyanate (1 : 300,000). Again, a series of electrodes which have been found to be in close agreement when

checked against a standard solution of ferrous and ferric chloride may show a surprising degree of variation in initial potential level when inserted into different tubes containing portions of the same milk, as indicated by the data shown in Table I. The range between highest and lowest readings is 111 mv., more than the difference between the initial levels of the two curves for Milk 1 (their Fig. 1), which Thornton *et al.* state "was immediately overpoised by the resazurin".

TABLE I
DIFFERENCES IN INITIAL POTENTIALS AMONG 12 ELECTRODES
IN SIX TUBES OF THE SAME MILK (JULY 24, 1941)

Tube No.	Electrode No.	Initial Eh, v.	Deviation from average, mv.
1	1	+0.274	-41
	2	+0.288	-27
2	3	+0.288	-27
	4	+0.331	+16
3	5	+0.385	+70
	6	+0.365	+50
4	7	+0.275	-40
	8	+0.305	-10
5	9	+0.309	-6
	10	+0.323	+8
6	11	+0.317	+2
	12	+0.317	+2
Average		+0.315	
Extremes of variation			111

In view of the unreliability of readings from single electrodes, we have invariably used duplicate electrodes¹ in each tube, and, wherever possible, duplicate tubes. When one electrode appears to be behaving peculiarly, its readings are discarded. By thus obtaining two or more sets of readings on each sample, and by running a wide variety of samples, we feel that we are less likely to have been misled regarding the shape of the time-potential curves for milk containing resazurin or methylene blue.

Almost all workers in the field of biological oxidation-reduction recognize the need for avoiding errors resulting from polarization. As Wilson *et al.* (34) have expressed it, "Quite fictitious potential values can be obtained unless this tendency to polarization due to drawing of current through the electrode is avoided, particularly if the milk is unstirred, as is essential in reproducing the conditions of the reduction test". Polarization is difficult to avoid with the type of apparatus used by Thornton *et al.* (32). In view of the current

¹ The electrodes and potentiometric set-up have been described in a previous paper (17).

general use of apparatus which minimizes polarization, the statement of these workers that "Oxidation-reduction potentials were measured by means of the usual electrometric set-up . . ." is likely to be misleading. Comparison of their curves with those obtained with more suitable apparatus (13, 16, 17, 34) suggests that polarization may indeed have influenced some of their readings. This, together with their reliance upon single electrodes, makes it difficult to dismiss the impression that the curves that they have published, and upon which they appear to base much of their objection to resazurin, are by no means truly representative.

The statement of Thornton *et al.* that "the time required to reach either the pink or white end-point was a function not only of bacterial influences but also of the comparative poisoning capacities of the milk and dye systems" suggests that differences in these poisoning properties may result in such marked discrepancies as to render the results of the resazurin test of questionable value. We have seen nothing to lead us to believe that such differences enter into the question. That such discrepancies rarely occur is also indicated by the close correlation between the resazurin (pink) and methylene blue reduction times (3, 15, 18, 19).

Discussing the possibility of shortening the reduction test by "overpoising" the milk, Thornton *et al.* state that "this appears feasible only if milks do not vary in their inherent reducing intensities and capacities". They believe "it is not unreasonable to assume varying poisoning in market milks" and that "interpretations of the behaviour in milk of such strongly poised systems as resazurin should be tempered with caution". No evidence is presented to substantiate this opinion, although reference is made to Greenbank's (12) work. Greenbank showed that when tested 6 to 48 hr. later, freshly drawn milks showed varying rises in Eh as a result of adding small amounts of copper. The difference between milks showing greatest and least change after six hours was 14 mv., which can scarcely be regarded as significant in relation to resazurin reduction. There was no indication that this had any bearing upon the point under discussion, i.e., that resazurin might "overpoise" certain milks. While we have seen nothing in our own studies to lead us to believe that variations in the poisoning properties of different milks are of sufficient magnitude to influence the results of the resazurin test, it seemed worth while investigating this point more fully. To date milks from 70 individual cows as well as 24 herd samples from different shippers have been studied.

We first attempted to show differences in initial potential and poisoning properties by measuring the change in potential 10 min. after the addition of 10 p.p.m. of copper, along the lines suggested by Fay *et al.* (6). (After the first few trials, however, we omitted adjustment of the pH of the milk to 6.35 as was done by these workers.) Changes in potential were very slight, the maximum noted being 12 mv.

We next studied the effect of added reducing agents, including cysteine, hydroquinone, sodium sulphite, and sodium thioglycollate. Fig. 3 depicts time-potential curves for six milks from individual cows showing varying

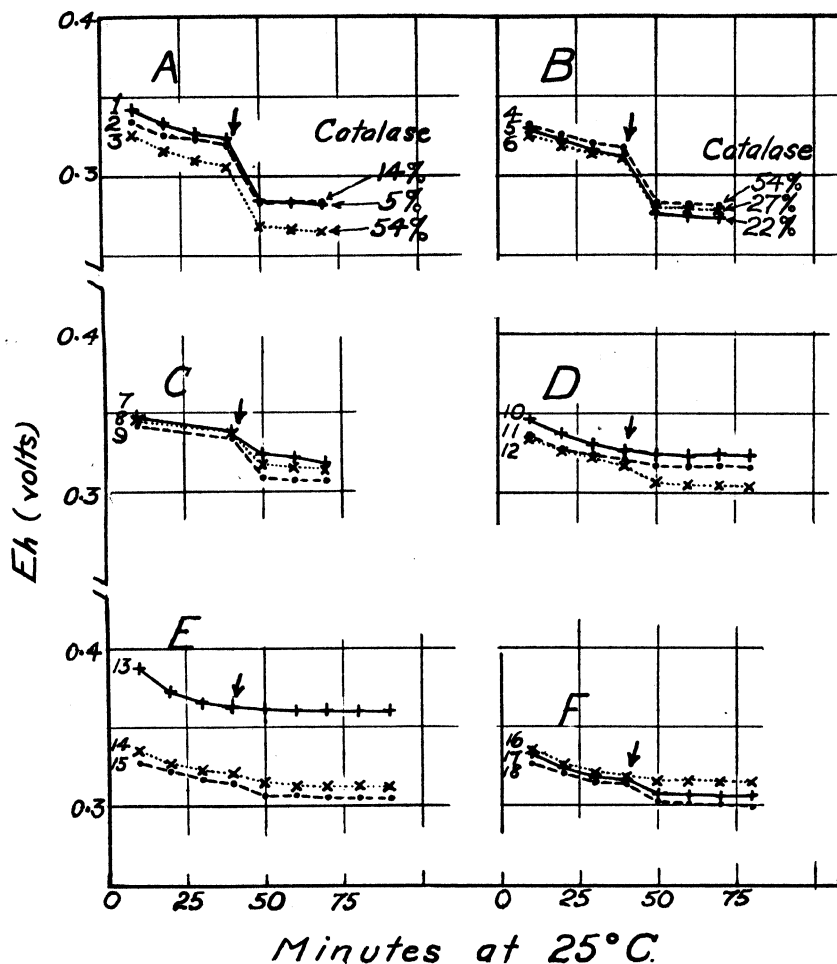


FIG. 3. Effect upon *Eh* of addenda of various reducing agents. Samples 1 to 6 = individual cow milks, 7 to 18 = herd milks. A and B: 1 ml. 2.5% solution of hydroquinone added at \downarrow ; C: 1 ml. 0.1% cysteine hydrochloride added at \downarrow ; D, E, and F: 1 ml. 0.01% cysteine hydrochloride added at \downarrow .

degrees of udder abnormality (A and B), as well as those for 12 herd milks (C to F). Each curve represents the average of readings from four electrodes. These milks, representing about one-fifth of the total number studied, show such slight differences in poisoning properties that it seems most unlikely that the results of the resazurin test would be affected thereby. This contention is supported by the data in Fig. 4 showing the effect of repeated addenda of cysteine to milks with and without resazurin. In no instance has the presence of resazurin in the usual concentration caused any downward distortion of the time-potential curve. In fact, as has recently been reported (16), the presence

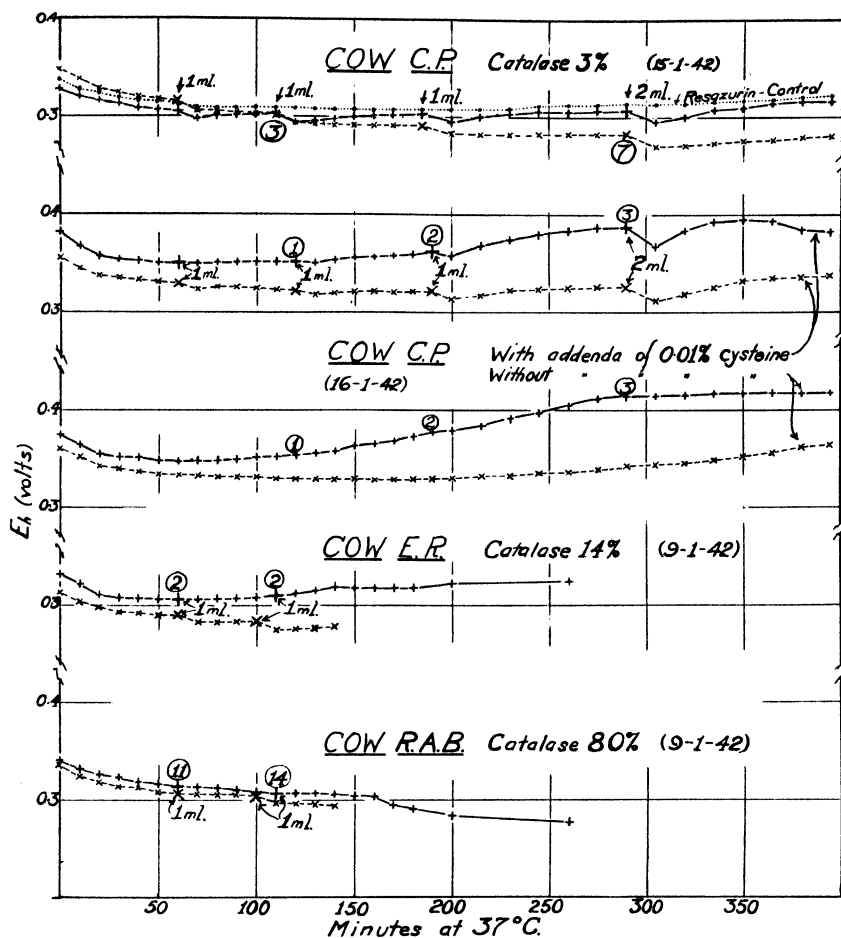


FIG. 4. Effect of addenda of 0.01% solution of cysteine hydrochloride upon the E_h of milks with and without resazurin. Ringed numbers represent resazurin colours (0 = initial, 16 = pink). +—+ = resazurin added; x—x = distilled water added.

of resazurin in normal milk generally results in a slight *upward* trend in the curve. This tendency is absent from the milk of Cow R.A.B., the abnormality of which is reflected in the much greater change in resazurin colour (as indicated by the ringed numbers) as well as in the slow downward trend of the curve.

Thornton *et al.* state "that some milks of low bacterial content, presumably abnormal, are rather strongly poised at E_h levels negative to the usual values of fresh milk". If this statement means that such milks show a lower initial level of E_h and a greater resistance to change of potential (stronger poisoning) than do normal milks, we are unable to agree. In Table II are shown E_h readings from a series of milks of varying degrees of abnormality.

TABLE II

Eh READINGS AND COLOUR CHANGES IN RESAZURIN-MILK MIXTURES INCUBATED AT 37° C.
PAIL SAMPLES FROM COWS IN C.E.F. HERD. BACTERIAL COUNTS (PLATE) <10,000 PER ML.

Sample No.	Leucocytes	Catalase, %	Eh reading, mv.					Resazurin colour No. after:			
			Initial	1 hr.	2 hr.	3 hr.	4 hr.	1 hr.	2 hr.	3 hr.	4 hr.
1	3,700,000		359	360	365	370	375	6	9	9	10
2	3,310,000		285	271	272	272	257	9	10	11	12
3	3,100,000		288	279	263	239	176	9	13	13	16
4	2,000,000		329	323	306	280	182	11	13	17	19
5	1,590,000	44	308	298	286	275	260		10	13	14
6	1,500,000		366	365	364	365	365	4	8	8	9
7	1,270,000		370	368	366	359	340	8	11	13	13
8	1,160,000	48	311	314	314	315	316	7	11	12	13
9	970,000	58	312	314	322	329	337	6	9	10	11
10	890,000	33	332	328	329	331	334		9	13	14
11	860,000		287	278	277	272	272	2	7	8	9
12	830,000		314	313	304	300	292	7	10	12	12
13	740,000	25	326	333	343	355	356	2	5	6	8
14	726,000	26	315	311	304	299	283	7	10	12	13
15	500,000		297	301	309	320	326	3	7	7	8
16	413,000	50	357	354	355	357	361		8	10	11
17	391,000		305	307	317	327	337	2	4	6	6
18	381,000	15	338	341	343	343	348		5	8	8
19	381,000	31	285	280	279	282	288		9	12	15
20	359,000		315	316	315	316	312	4	6	7	10
21	342,000	19	294	299	284	286	293	3	5	8	9
22	300,000		339	339	344	348	356	6	7	10	10
23	211,000		313	314	317	322	327	4	6	6	6
24	159,000	14	358	353	349	352	349		4	7	7
25	123,000	7	318	317	320	324	328	3	4	4	6

These data fail to show any marked difference in initial Eh level correlated with leucocyte content or catalase value; on the other hand, they indicate, as do also the curves recently presented in another paper (16), that milks with high leucocyte content sometimes, but not invariably, show a slow downward drift of potential long before bacterial numbers are sufficient to account for the reduction. (See also curves for Milks VI and VIII, Fig. 1, and for Cow R.A.B., Fig. 4.) We are unable to interpret these data as meaning that such milks are more strongly poised than normal milks.

The findings reported above, supplementing our experience with many hundreds of samples in the various modifications of the resazurin test, leave us with the conviction that differences in poisoning properties among different milks are too slight to have any significant influence upon the results of the test.

Significant differences in initial potential levels of milk have been reported by various workers. Thornton and Hastings (28) stated that "the positive Eh limits of all the milks examined lay between +0.2 and +0.3 volt. This corresponds closely to the limits reported by Clark and others for milk and some other biological fluids." Among others who have reported similar

potential levels are Fay and Aikins (5), Gebhardt and Sommer (9), Greenbank (12), Gould (10), Tracy *et al.* (31), Jackson (14), and Thornton *et al.* (29, 30). On the other hand, we have almost always obtained initial potential values for market milks of between $+ 0.3$ and $+ 0.4$ v. Similar levels have been reported by Wilson *et al.* (34), Hobbs (13), Gould (11), Swanson and Sommer (25), Garrett (8), and Webb and Hileman (33). Wide variations in initial potential for samples from individual quarters ($+ 0.23$ to $+ 0.38$ v.) have been reported by Webb and Hileman, while Swanson and Sommer observed a relationship between the reduced ascorbic acid content and the potential of milks from individual cows. Since the higher levels of potential for market milks have been reported by workers employing vacuum tube potentiometric circuits, while the older type of apparatus was used by all of the group reporting lower levels, we have measured the potentials of a series of six samples from individual cows and one vat sample concurrently, using both Leeds and Northrup Type K and Beckman Model G (vacuum tube) potentiometers. Although time-potential curves from the former apparatus showed slight fluctuations, apparently due to polarization, the Eh values for both sets of readings were never more than 10 mv. apart. It seems unlikely therefore that these differences in level of initial potential are attributable to the methods of measurement employed. A study of our own data, as well as that of Webb and Hileman, shows no appreciable seasonal differences. We are therefore unable to suggest any explanation for the marked differences referred to above.

Conclusions

Compared to methylene blue, resazurin is much more sensitive to milk from diseased or otherwise abnormal udders. It is equally as sensitive to the reducing activity of bacteria growing in the milk. This greater sensitivity to abnormal milk is regarded as a distinct advantage since such milks can scarcely be regarded as first-class products.

Our findings fail to support the view of Thornton *et al.* that resazurin exerts such a strong poisoning effect as to complicate the use of this dye or the interpretation of results. There is also no evidence that differences in poisoning properties of different milks are of sufficient magnitude to affect the results of the test.

Acknowledgments

The author is indebted to Dr. I. Hlynka for helpful suggestions in the preparation of this paper, also to Mr. J. B. Fischer for assistance with some of the potentiometric determinations reported.

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Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 20, SEC. C.

JULY, 1942

NUMBER 7

SECONDARY SPORES IN *CORTICIUM EFFUSCATUM*¹

BY MILDRED K. NOBLES²

Abstract

Corticium effusatum Cooke & Ellis is heterothallic and tetrapolar. Oedoe-phaloid conidiophores bearing uninucleate conidia are developed on both haploid and diploid mycelia. Conidia produced on a haploid mycelium germinate only rarely to give new mycelia, but they are able to diploidise a suitable haploid mycelium when in association with it. A small percentage of the conidia produced on a diploid mycelium germinate to give haploid mycelia, part of which are identical in pairing reactions with one parent, the remainder with the other parent. Chlamydospores are produced on haploid and diploid mycelia and reproduce the generation on which they are borne.

Introduction

The production of conidia by *Corticium effusatum* Cooke and Ellis in culture was admirably described and illustrated by Lyman (10). This work, however, predated the modern knowledge of sexuality in the Hymenomycetes, first stated by Bensaude (1) and Kniep (9), and lacks certain information regarding interfertility phenomena and distribution of nuclei, without which it is impossible to obtain an adequate understanding of the life history of the fungus, and in particular of the function of the conidia. The present study was undertaken to determine these additional facts.

Cultural Studies

Haploid Mycelium

Cultures were obtained from two fruit bodies, collected by Dr. R. F. Cain, the first (University of Toronto Herbarium No. 6503) on *Alnus incana* at Holland River Marsh, Ont., October 3, 1934, the second (U. of T. Herbarium No. 6502) on wood of a deciduous tree near New Durham, Ont., October 29, 1934. Both sporophores yielded heavy deposits of globose basidiospores, 5.3–7.0 μ in diameter. These, contrary to Lyman's experience, germinated readily in distilled water, 2% malt extract solution, and on agar containing 2% malt extract, producing a vesicle which either elongated directly or branched immediately, leaving the spore at the side of the germ tube. After the sporling had achieved a considerable size, with several branches, simple septa appeared; the formation of this type of septum has continued in mycelia

¹ Manuscript received March 30, 1942.

Contribution from the Department of Botany, University of Toronto, Toronto, Ont. Part of a thesis submitted to the School of Graduate Studies, University of Toronto, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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arising from single basidiospores during the more than seven years that these cultures have been maintained.

On medium containing 2% malt extract and 2% agar the mycelium from a single basidiospore grows slowly, producing a white mat, at first scanty and consisting of scattered fibres, later more plentiful, the newer growth raised, cottony, tufted, the older mycelium collapsed and woolly, or farinaceous because of the masses of conidia.

Conidia

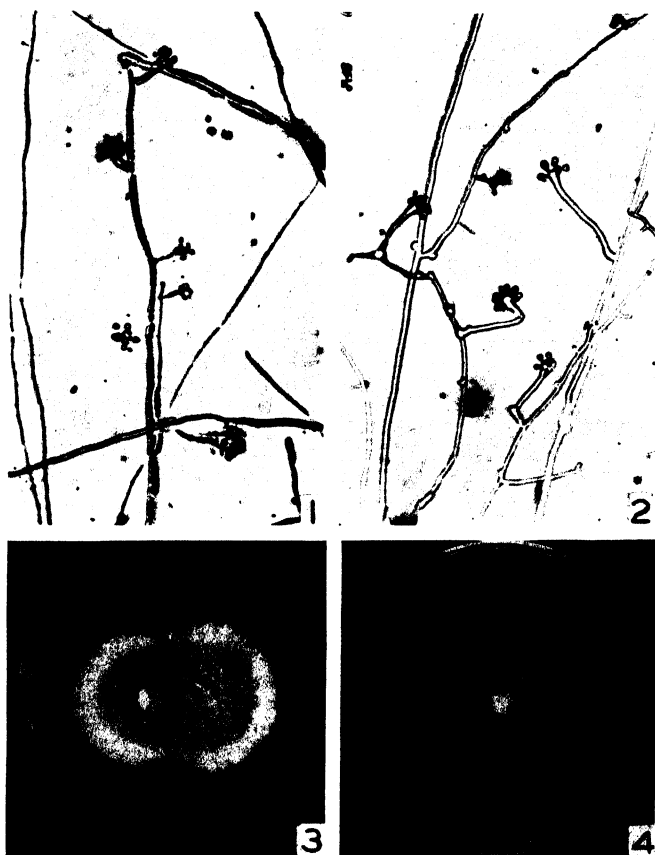
The formation of conidia begins within two or three days after the germination of the basidiospores or transfer of the mycelium to fresh agar, and continues throughout the life of the mycelium. As in *Fomes annosus* (Fries) Cooke (*Trametes radiciperda*), the conidiophores of which were first described by Brefeld (4) and subsequently by many other authors, and in *Peniophora Allescheri* Bres. studied by the author (12), the conidia are borne on oedoccephaloid conidiophores (Fig. 1) which are abundant on all aerial hyphae, occurring singly or in coremium-like aggregations. The heads are globose, 6.5–10.0 μ in diameter, the distal portion being covered with the slender, tapering sterigmata, each bearing a conidium. The conidia are usually crowded on the conidiophore, their number ranging from 2 to 30 per head, but averaging about 20. Lyman (10, p. 178 and Plate 21, Figs. 87 to 91) has described and illustrated the successive stages in the development of a conidiophore and its spores, and has noted the proliferation and branching of conidiophores, which is frequently observed. Following the formation of conidia, the conidiophores and cells from which they arise appear vacuolate and finally empty. The conidia are hyaline, thin-walled, globose like the basidiospores but smaller in size, 2.3–3.8 μ in diameter, averaging 2.75 μ . No spore deposit is produced from mycelia bearing conidia, so it must be assumed that the conidia, unlike the basidiospores, are not projected forcibly from the sterigmata.

Chlamydospores

Numerous chlamydospores occur on the aerial and submerged hyphae of all monosporous mycelia. These are produced in an intercalary or terminal position by the rounding up of a portion of a cell, the concentration of the cytoplasm in that enlarged part, and its enclosure within a wall. The chlamydospores are broadly ovoid to lemon-shaped, with walls slightly thickened, 7.5–18.0 \times 7.5–16.5 μ .

Pairings of Monosporous Mycelia

Five weeks after their isolation, 24 mycelia, each derived from the germination of a single basidiospore from fruit body No. 6503, were grown together in pairs in all possible combinations and the resulting mycelia examined for the presence of clamp connections. The results are shown in Fig. 5, in which a plus sign indicates the occurrence of clamp connections, a minus sign their absence. It is evident that the mycelia fall into four groups, the members of one group reacting with the members of only one other group in such a way



FIGS. 1 TO 4. *Corticium effuscatum*. FIG. 1. Haploid hyphae and conidiophores. $\times 400$. FIG. 2. Diploid hyphae and conidiophores. $\times 400$. FIG. 3. Pairing of two compatible haploid mycelia 10 days after inoculation. $\times 0.5$ approximately. FIG. 4. Haploid colony diploidised by means of suitable conidia. $\times 0.5$ approximately.



	AB				ab								aB				Ab							
	1	13	17	4	7	9	10	15	16	19	20	21	22	2	6	8	18	24	3	5	11	12	14	23
AB	1	—	—	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—
	13	—	—	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—
	17	—	—	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—
	4	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
ab	7	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	9	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	10	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	15	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	16	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	19	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	20	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	21	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
aB	22	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	+
	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	+
	8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	+
	18	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	+
Ab	24	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	+
	3	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	—	—	—	—	—	—
	5	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	—	—	—	—	—	—
	11	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	—	—	—	—	—	—
	12	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	—	—	—	—	—	—
	14	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	—	—	—	—	—	—
	23	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	—	—	—	—	—	—

FIG. 5. *Corticium effuscatum*. Results obtained by pairing in all possible ways 24 mycelia, each derived from the germination of a single basidiospore.

as to form mycelium bearing clamp connections. Hence *Corticium effuscatum* is heterothallic and of the tetrapolar type of interfertility reaction. Where tetrapolarity occurs it has been assumed that the interfertility reactions are determined by two pairs of factors which are designated by the symbols *Aa* and *Bb*. The mycelium originating from the germination of a single basidiospore will then contain nuclei of one of the following constitutions *AB*, *ab*, *Ab*, *aB*. Two mycelia, which when grown together produce a mycelium with clamp connections, must contain nuclei of complementary constitution, *AB* + *ab* or *Ab* + *aB*. Thus in Fig. 5 the formula *AB* has been assigned arbitrarily to Mycelia 1, 13, and 17, from which it follows that Mycelia 4, 7, 9, 10, 15, 16, 19, 20, 21, and 22 must be designated by the formula *ab* and the other two groups by *aB* and *Ab* as shown. These results were corroborated by a second series of pairings of 16 cultures, each derived from a single basidiospore of fruit body No. 6502. Here again the mycelia could be divided into four groups.

Four haploid mycelia of collection No. 6503, one of each reaction group, were paired with four corresponding mycelia from collection No. 6502, and the resulting mycelia examined for the presence of clamp connections. The findings are incorporated in Fig. 6, from which it is seen that clamp connections were formed in every pairing. This indicates that in *Corticium effuscatum*, as in many other species in the Hymenomycetes, which have been studied in a

similar manner, complete interfertility obtains when monosporous mycelia derived from fruit bodies from different localities are grown together.

		6503			
		1 7 2 3			
6502	3	+	+	+	+
	1	+	+	+	+
	2	+	+	+	+
	4	+	+	+	+

FIG. 6. *Corticium effuscatum*. Results obtained by pairing four haploid mycelia from fruit body No. 6502 with four haploid mycelia from fruit body No. 6503.

Diploid Mycelium

Diploidisation of compatible haploid mycelia takes place fairly rapidly. Fig. 3 is a photograph taken 10 days after pairing of Mycelia 1 and 9 from collection No. 6503 (Fig. 5). It may be observed that there is a complete intermingling of the two mycelia at the line of meeting, and that the more abundant and fluffy diploid mycelium composed of hyphae bearing clamp connections, has formed an evident border around the colonies.

When subcultured on malt extract agar, the diploid mycelium forms a slightly more vigorous colony than the haploid, as shown by the formation of more plentiful aerial mycelium and the more rapid increase in the diameter of the colony. Microscopically the diploid mycelium resembles the haploid in production of chlamydospores and conidia but differs by having a clamp connection at every septum. The conidiophores and conidia are indistinguishable from those borne on haploid mycelium (Fig. 2).

When about five or six weeks old, such diploid colonies form fruiting surfaces similar to fructifications produced in nature. No detailed study of their development was made, but it was observed that basidia and gloecystidia are produced in abundance and quantities of basidiospores are formed which are shot from the sterigmata in the usual way, giving heavy spore deposits. It would appear, however, that the mechanism for spore discharge frequently fails to function in this species, with the result that many basidiospores remain on the surface and become embedded by the subsequent proliferation of the hymenial layer. These embedded spores, an important diagnostic character of *Corticium effuscatum*, were termed "chlamydospores" by Burt (7), although Lyman (10) had described them earlier as "discharged basidiospores which are always imbedded in the growing hymenium". That they are basidiospores is borne out by the observation of the close agreement in size and shape between the embedded spores and basidiospores attached to basidia and in spore deposits, and by the fact that in young fruiting layers in culture, sufficiently loosely arranged to allow for careful microscopic examination of the component parts, the embedded spores are never attached to hyphae, in the manner in which chlamydospores occur, nor are they of the size and shape of the true chlamydospores of this species. The "antler-like" hyphae (dichophyses) usually present in fruit bodies of *C. effuscatum*, and which are the

basis for the transfer of this species to the genus *Asterostromella*¹ by Bourdot and Galzin (3) were not noted in cultures, although they may have escaped notice since no prolonged search for them was made.

Cytological Studies

A cytological examination has been made of basidiospores, sporelings, haploid and diploid mycelium, chlamydospore formation and stages in the development of conidiophores and conidia, using the technique previously described by the author (12).

Basidiospores

Basidiospores scraped from a spore deposit and mounted directly in Bouin's solution were found, on staining with Haidenhain's haematoxylin, to be binucleate (Fig. 9). Although it has not been demonstrated cytologically it seems clear from the experimental results set forth above that the two nuclei are of the same genetic constitution, and are presumably sister nuclei, derived from a mitotic division of the single nucleus that entered the spore during its formation.

Haploid Mycelium

On germination the two nuclei of the spore migrate into the vesicle (Fig. 10), which elongates (Fig. 11). Nuclear divisions follow to give the four-nucleate (Fig. 12) and finally, many-nucleate stages. Septum formation is delayed so that the young sporeling contains several nuclei (Fig. 13) with relatively few septa and these appear to be formed without reference to the nuclei. This multinucleate condition persists throughout the life of the haploid mycelium. Terminal cells in actively growing cultures have been observed to contain up to nine nuclei, while cells at a distance from the tip have been seen with one, two, or more nuclei.

Chlamydospores

Chlamydospores are produced in abundance in haploid hyphae, and in stained preparations are very prominent since their dense cytoplasm retains the stain after it has been removed from both the cytoplasm and nuclei in vegetative hyphae. During their formation, a small amount of vacuolate cytoplasm remains in the cells (Figs. 14 to 16) but when development is complete the cells appear empty. From one to eight nuclei have been observed in chlamydospores and since no divisions have been seen, it seems probable that they contain only the nuclei that were present in the cell in which they originated. This is substantiated by the observation that the chlamydospores containing many nuclei (Fig. 16) are always formed from multinucleate terminal cells.

¹ As pointed out by G. W. Martin (Iowa Acad. Sci. Trans. 44: 48, 1938) the genus *Asterostromella* v. Höhn. & Litsch. is antedated by *Vararia* Karst. of which *Corticium investiens* (Schw.) Bres. is the type species. If the presence of these dichophyses, together with the other characters, can be shown to indicate relationship with other species, *C. effuscatum* should perhaps be included in *Vararia*. Since no combination is yet available, it has seemed best for the purposes of this paper to retain the species in *Corticium*.

Conidia

A developing conidiophore can be distinguished from a branch by its broad base and more deeply staining cytoplasm (Fig. 17). The initial number of nuclei in a conidiophore formed on haploid mycelium is the same as that in the cell from which it developed. These migrate to the tip of the conidiophore and there divide (Figs. 18 to 19). Conidiophores have been seen in which nuclear divisions had been in progress when fixation occurred but figures of these have not been included because of the very small size of the nuclei. Following each division the nuclei appear successively smaller and after the 8- or 12-nucleate stage it is no longer possible to ascertain the exact number and position of nuclei within the head. However the nucleus can be seen more clearly after its migration into the spore, and it is apparent that the conidia are uninucleate.

Diploid Mycelium

The cells of the hyphae bearing clamp connections are consistently binucleate (Fig. 20), the nuclei dividing through the agency of the clamp connections. Chlamydospore formation proceeds as in the haploid mycelium, the chlamydospore always containing the two nuclei of the dikaryon (Figs. 21, 22).

The stages in the development of conidiophores on the diploid mycelium are similar to those in haploid mycelium. The young conidiophore, recognizable by its enlarged base and deeply staining contents, receives the two nuclei of the parent cell (Fig. 23). These divide (Fig. 24) without the intervention of a clamp connection, to give a four-nucleate structure (Fig. 25), the nuclei of which divide successively to give eight (Fig. 26) and 16 or more nuclei. As in the haploid conidiophore it is not possible to count the nuclei definitely beyond the eight-nucleate stage, but after the conidia are mature it is possible to distinguish *one nucleus* in each conidium (Fig. 27). It seems evident from these cytological observations, taken together with the experimental results reported below, that the binucleate clamp-bearing mycelium produces conidiophores in which the nuclei of the dikaryon, without preliminary fusion, divide successively to provide numerous nuclei, one of which migrates into each conidium. As in *Peniophora Allescheri*, studied by the author (12), the binucleate mycelium gives rise to uninucleate conidia.

Germination of Chlamydospores and Conidia

Chlamydospores

No special study has been made of germinating chlamydospores beyond the observation that they germinate very readily and vigorously. Twenty-four hours after being sown in hanging drop cultures of malt extract solution they had produced sporelings of considerable size, with several cells. Chlamydospores produced on a haploid mycelium germinate to give mycelia identical with it; those produced on a diploid mycelium give rise to hyphae bearing clamp connections immediately.

Conidia

First attempts to procure germination of conidia from a haploid mycelium in hanging drop cultures of malt extract solution met with complete failure. Observations were continued for nine days, during which the conidia remained unchanged. Lyman (10) encountered similar difficulties but observed (p. 181) that "the presence of growing hyphae appears to exercise a stimulating influence on the conidia". This suggested that they might act as diploidising agents, a function that Brodie (5, 6) has described for the oidia of *Coprinus lagopus* and *Collybia velutipes*. To test this the following experiment was carried out.

A haploid mycelium was allowed to grow on malt extract agar in a Petri dish for four days, the colony then being approximately 2.5 cm. in diameter. At that stage a drop of a suspension of conidia from a compatible haploid mycelium was placed at each of three marked spots on the periphery of the colony, either on the tips of the hyphae or directly in their path. Two days later it was observed that the growth of the colony had been retarded in the areas where the conidia had been added, so that noticeable bays were apparent around the circumference of the colony (Fig. 4). Microscopic examination revealed that many of the conidia had short germ tubes, and that diploidisation had occurred, as shown by the presence of clamp connections on the hyphae in the vicinity of the added conidia. This experiment was carried out with three pairs of compatible haploid mycelia, in triplicate, and in every plate hyphae with clamp connections were formed within 72 hr. of the addition of the conidia. Hence it was concluded that conidia borne on a haploid mycelium can germinate in the presence of hyphae and that the sporelings so produced are able to diploidise a suitable haploid mycelium.

A second series of experiments was carried out to test the ability of conidia produced on diploid mycelium to diploidise haploid mycelia. Colonies of basidiospore culture No. 1 from No. 6503 (Fig. 5) were grown on malt extract agar in Petri dishes until they had reached a diameter of approximately 2.5 cm., and drops of a suspension of conidia from the diploid mycelium No. 1 \times 9 were added at marked areas on the peripheries. Clamp connections appeared on hyphae of the original colonies in the vicinity of the added conidia within 72 hours. This was repeated with basidiospore culture No. 9 (Fig. 5). At this time the sporelings originating from the germination of the conidia were minute in size, so that the diploid mycelium that appeared could not have resulted from their germination, but only from the diploidisation of the original haploid colony. It seems evident that conidia produced on a diploid mycelium are capable of diploidising both the haploid mycelia that entered into the formation of the diploid mycelium.

In later experiments it was found that a few conidia germinated when sown on malt extract agar, the method of germination resembling that described for basidiospores. To evaluate the spores produced on a diploid thallus, conidia from the diploid mycelium No. 1 \times 9 (Fig. 5) were sown on agar in Petri dishes, and although relatively few spores germinated, 17 single

conidium cultures were isolated. These produced haploid mycelia which were indistinguishable from those from single basidiospores. This verified the cytological observation that the conidia on diploid mycelium are uninucleate.

These 17 mycelia were grown together in pairs in all possible combinations and the resulting mycelia examined for the presence of clamp connections. The results are incorporated in Fig. 7, from which it may be observed that the mycelia fall into *two* groups, a member of one group reacting with a member of the other group in such a way as to produce mycelium bearing clamp connections.

Each of the single conidium cultures was then paired with each of the parent basidiospore cultures, that is, 1 and 9 from Fig. 5, and the resulting mycelia examined for the presence of clamp connections. The results are summarized in Fig. 8 from which it is evident that single conidium cultures Nos. 1, 2, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, and 17 form diploid mycelium when mated with basidiospore culture No. 9, and so belong to the group designated by the formula *AB* in Fig. 5 while conidium cultures Nos. 3, 6, and 16 react with basidiospore culture No. 1 and so belong to the group *ab*.

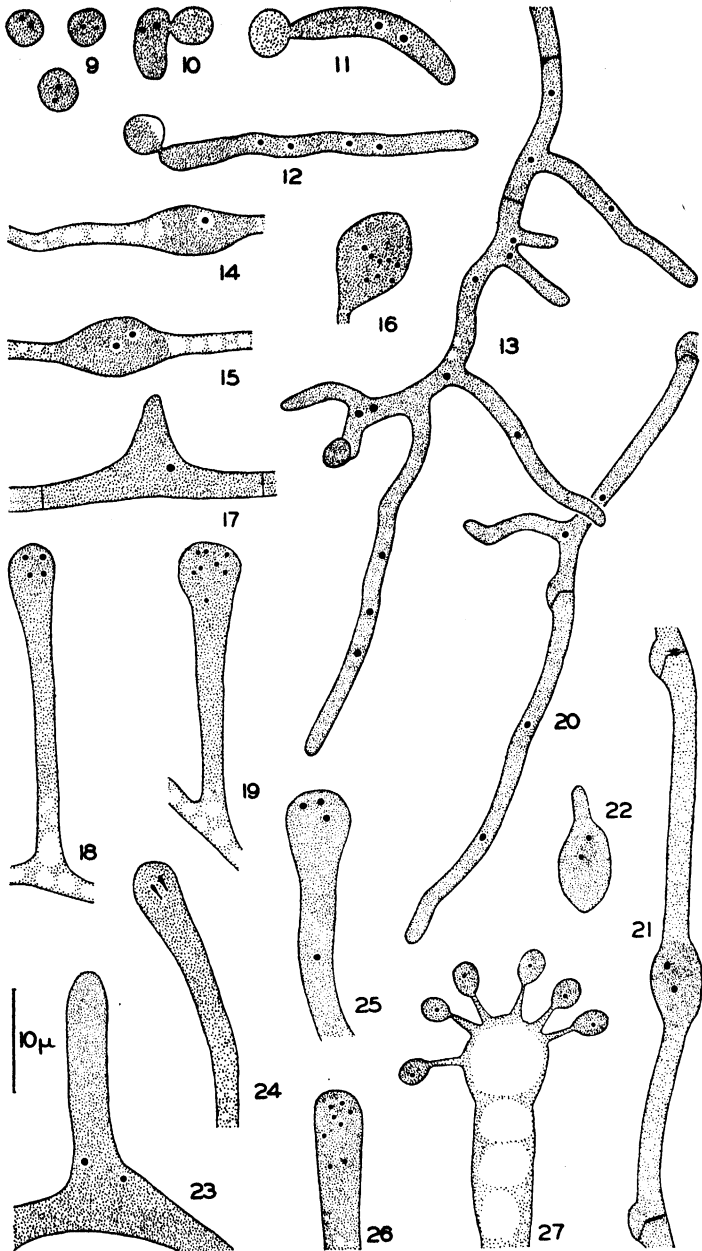
		AB														ab		
		1	2	4	5	7	8	9	10	11	12	13	14	15	17	3	6	16
AB	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+
	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+
	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+
	5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+
	7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+
	8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+
	9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+
	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+
	11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+
	12	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+
	13	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+
	14	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+
	15	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+
	17	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+
	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
ab	6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—

		1	9
AB	1	—	+
	2	—	+
	4	—	+
	5	—	+
	7	—	+
	8	—	+
	9	—	+
	10	—	+
	11	—	+
	12	—	+
	13	—	+
	14	—	+
	15	—	+
	17	—	+
ab	3	+	—
	6	+	—
	16	+	—

FIGS. 7 AND 8.

FIG. 7. *Corticium effuscatum*. Results obtained by pairing in all possible combinations 17 mycelia, each derived from the germination of a single conidium produced on a diploid mycelium. FIG. 8. *Corticium effuscatum*. Results obtained by pairing the 17 mycelia of Fig. 7 with each of the parent mycelia.

All the conidia produced on the diploid mycelium No. 1 \times 9 are haploid in value and of only two types, as determined by their pairing reactions, and the two types are those of the two parent cultures. Hence, no segregation of factors, such as takes place in a basidium, occurred during the formation of the nuclei that entered the spores, that is, no fusion of the nuclei of the dikaryon preceded the nuclear divisions in the conidiophore. Thus the cytological observations recorded above are substantiated by the results of culture work.



FIGS. 9 TO 27. *Corticium effuscatum*. FIG. 9. Mature basidiospores. $\times 1400$. FIGS. 10 TO 12. Stages in germination of basidiospores. $\times 1400$. FIG. 13. Young haploid mycelium. $\times 1000$. FIGS. 14 TO 16. Chlamydospores produced on haploid mycelium. $\times 1000$. FIGS. 17 TO 19. Stages in conidiophore development on haploid mycelium. $\times 1400$. FIG. 20. Binucleate clamp-bearing hypha. $\times 1000$. FIG. 21. Formation of chlamydospore in binucleate cell. $\times 1000$. FIG. 22. Mature chlamydospore. FIGS. 23 TO 27. Stages in conidiophore development on diploid mycelium. $\times 3000$.

Discussion

Corticium effuscatum bears uninucleate conidia on oedocephaloid conidiophores on both haploid and diploid mycelia. While the production of oidia capable of repeating the haploid generation has been described for several species of Hymenomycetes, spores borne on organized conidiophores have been recorded for only a few species, including *Fomes annosus* (*Trametes radiciperda*) by Brefeld (4), *Peniophora Allescheri* by the author (12), *Pleurotus corticatus* by Kaufert (8), and *Corticium effuscatum* by Lyman (10).

Also, a few species are known in which uninucleate spores are produced on the dikaryotic mycelium. In most of the species this has been achieved by a reversion to the haploid condition of parts of the dikaryotic mycelium through the separation in a hypha of the nuclei of a dikaryon, and the subsequent growth of monokaryotic mycelium with the characteristics of such mycelium, including that of spore production. This was observed in cultures of *Corticium alutaceum*¹ by Lyman (10) and of *Collybia velutipes* by Brodie (6).

In *Pholiota aurivella*, Martens and Vandendries (11) observed that conidia borne on a diploid mycelium at first contain two nuclei which are later separated by a wall so that the mature spore is composed of two uninucleate cells. This may be interpreted as a return to the haploid condition within the spore. This haploid state may be very brief, ending with the disappearance of the septum and the germination of the spore, now binucleate, to give a dikaryotic mycelium; or the haploid condition may be continued as a result of the disjunction of the spore at the septum, with the germination of each uninucleate half to produce a haploid mycelium. Thus, in *Pholiota aurivella* there is a provision for a return to the haploid condition through structures borne on true diploid hyphae.

In *Peniophora Allescheri* described by the author (12) and in *Corticium effuscatum*, the conidiophores are borne on diploid mycelium and each originally contains a dikaryon, the nuclei of which divide simultaneously and successively until the head contains many nuclei, known to be of two kinds as to genetic constitution, but no longer existing in conjugate pairs. The subsequent migration of one nucleus into each conidium results in the formation of uninucleate spores on dikaryotic mycelium, a phenomenon rarely encountered in this group of fungi.

In *Corticium effuscatum* these conidia have lost to a marked extent their ability to germinate to give new individuals, at least under the conditions afforded them in the present investigation. Lyman (10) reached the same conclusion, writing (p. 181) "From his experiments the writer inclines to the belief that the conidia have partially lost the power of germination, and that they are not now of great importance in the reproduction of the plant". The present study has shown, however, that the conidia still retain an important

¹ It is evident from Lyman's description and figures (10) that the species that he describes was one quite different from *C. radiosum* Fries (= *C. alutaceum* (Schrad.) Bres.). As suggested by Biggs (2) it is probable that Lyman was dealing with a form of *C. coronilla* v. Höhn. & Litsch.

property in that they are able to bring about the diploidisation of suitable haploid mycelium.

Acknowledgments

The research here reported was conducted under the direction of Professor H. S. Jackson, of the Department of Botany, University of Toronto, to whom the author is indebted for stimulating suggestions and continued interest. She also wishes to thank Dr. R. F. Cain for supplying the specimens from which the cultures were obtained.

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POLARIZATION AND PROGRESSION IN PAIRING

I. INTERLOCKING OF BIVALENTS IN *TRILLIUM ERECTUM* L.¹

BY STANLEY G. SMITH² AND E. ROGER BOOTHROYD³

Abstract

A total of 346 interlocks, occurring in five plants, were classified as to type of interlocking and type of bivalent concerned. Two main types of interlocking, true and false, occurred in equal numbers, the interlocking involving different bivalents and also the two arms of one bivalent. An interlocked chiasma, the first reported case in which the chromatids could be traced, and a clear example of chromatid interlocking were observed. No correlation was found between the frequency of interlocking and the temperature at which meiosis occurred.

The ratio of the frequencies with which the individual bivalents were involved in interlocking is proportional to the relative lengths of the bivalent arms minus a minimum length. Interlocking of bivalents in the 10 possible combinations in pairs occurred with the frequencies expected from the frequencies with which the individual bivalents were involved.

All loops of the bivalents were involved in interlocking, odd and even loops being involved in the ratio of 3 : 1. That adjacent to the centromere and the most distal loops were most frequently concerned and centric interlocking occurred with a frequency only one-third that expected on a random basis. The loops involved were larger than corresponding loops free from interlocking.

Interlocking was found to reduce the chiasma frequency. The chiasma frequency of encircled bivalents was higher than that of bivalents in cells devoid of interlocks, which, in turn, had a higher frequency than the encircling bivalents of false interlocks. The factor causing the reduction was found to reduce the chiasma frequency of non-interlocked bivalents accompanying interlocked bivalents. The reduction in chiasma frequency is insufficient to have resulted from loss of chiasmata. It is proportional to the increase in size of the interlocking loops, which is the result, not of increased repulsion, but of simple interference with the positions in which chiasmata are formed at pachytene.

It is concluded that interlocking is a result of the derangement of chromosomes that are normally polarized and have their proximal parts in an orderly spatial arrangement prior to pairing.

Introduction

In chromosome mutants of hexaploid wheats (*Triticum vulgare* Host) it was noticed (18) that in a number of cells two or more bivalents were interlocked at meiotic metaphase. The percentage of cells containing such interlocked bivalents and the number of bivalents interlocked in these cells is unusually high in a speltoid mutant having only 40 chromosomes instead of the usual 42. Other speltoid mutants which have either 41 or 42 chromosomes with a homozygous deficiency show progressively fewer cases of interlocking. The heterozygous speltoids, which have either 41 or 42 chromosomes (numerically and segmentally deficient respectively), have a still lower frequency of interlocking again both as regards percentage of cells and percentage of chromosomes (preliminary unpublished data of Mrs. E. R. Sansome). In the normal *T. vulgare*, interlocking is extremely rare.

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Though a positive relationship clearly exists between degree of chromosome unbalance and frequency of interlocking, its cause could not be determined from observations at metaphase. But when during the making of smear preparations of *Trillium erectum* L. certain slides were found which contained a high percentage of interlocked bivalents, it appeared likely that their study might throw some light on the question of interlocking in wheat and the mechanics of chromosome pairing in general.

Triticum vulgare, besides being a hexaploid and possessing a large number of chromosomes which are individually indistinguishable, has chromosomes which it has not been possible to stain consistently so as to reveal their internal structure. *Trillium erectum*, on the other hand, is a diploid with only five pairs of chromosomes, each of which is morphologically distinct, and these can be readily stained to show the interrelationships of the four chromonemata of a bivalent. The present investigation was therefore undertaken in the hope of being able to discover some of the factors concerned with, and conditions resulting from, the interlocking of non-homologous bivalents.

Mechanics of Interlocking

As early as 1906 bivalent interlocking had been seen in *Salamandra maculosa* by Schreiner and Schreiner (see Belar, 3), and since that time it has been noted by many workers in a number of different organisms.

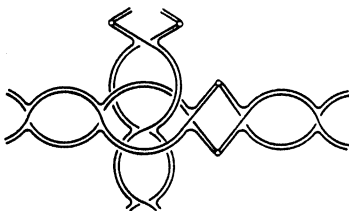


FIG. 1. *The relationship of the chromatids in a true interlock.*

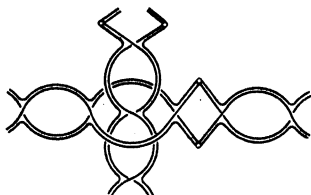


FIG. 2. *The relationship of the chromatids in a false interlock.*

When two homologues come together at zygotene, one or both members of another pair may be caught between them, resulting, with the formation of the necessary chiasmata, in "true" or "false" interlocking respectively (Figs. 1 and 2). Such interlocking has been seen at pachytene in *Dendrocoelum* (15), *Viviparous* (3), *Agapanthus* (9), *Dasyurus* (20), *Tulipa* (33), and in *Neodiprion* and *Chorthippus* (Smith, unpublished data). The interlocking of

non-homologous bivalents is therefore thought to be the result of a mechanical accident occurring during zygotene pairing (5, 14, 7, 23).

Dark (8) working with *Paeonia*, and Upcott (33) with *Tulipa*, found interlocking to be far more common in the tetraploid than in the diploid. One possible explanation given by Dark is that crowding of the chromosomes in the tetraploid would increase the chance of an interlock occurring. Upcott stresses the "large size of the nucleus" as well as "the large number of chromosomes present in the tetraploids", evidently implying that, relative to the diploid, there is need for more time in which to complete pairing.

Sax and Anderson (30, 31) suggest that bivalent interlocking may not be entirely mechanical in origin, but may be dependent upon the previous interchange of very short segments, the pairing of which would cause intertwining and subsequent interlocking of the chromosomes; these interchanges would be too small to be morphologically distinguishable. Dark (8) agrees that "true" interlocks occur at zygotene, but suggests that the "false" type may be the result of chromosome movements after zygotene. In *Eremurus spectabilis* Upcott (32) found false interlocking rare at metaphase compared with diplotene and diakinesis. She explains this as probably due to the bivalents slipping apart during metaphase orientation. Considering chromosome repulsion this seems more probable than that one should get pushed through another as Dark suggests.

In all the cases of bivalent interlocking which he noted in the tetraploid *Paeonia*, Dark found that the chromosomes were morphologically similar. This suggested to him that they might be homologous, and have been sufficiently closely associated to interlock either (a) through secondary pairing, or (b) through pachytene pairing to form a quadrivalent which did not persist, however, owing to failure to form the necessary chiasmata.

With regard to the position of interlocking, Mather (23) states that if pairing occurs at random along the chromosomes the position of interlocking should be random, and if there is little terminalization of chiasmata it should remain so. But should pairing start at the ends of the chromosomes interlocking would be most frequent in the central region and consequently if the centromere is median or submedian most of the interlocking would be in the centric loop. However, if interlocking has no influence on chiasma formation, as Mather (23) postulates, the position of interlocking would presumably be dependent solely on the chiasma frequency regardless of the method of pairing. At pachytene in *Agapanthus umbellatus*, Darlington (9) observed interlocking which usually coincided in position with the centromeres. This he suggested may result from pairing starting at the ends and pushing interlocks to the centromeres. The type of interlocking was difficult to ascertain, but from the absence of interlocks at metaphase most of it was thought to have been false. This supposition regarding the ultimate position assumed by interlocks is, however, not in agreement with most observations (Gelei *et al.*, see above); nor is that regarding the possible fate of false interlocks in agreement with our observations (see later, p. 376).

Catcheside (5), working with a mutant variety of *Oenothera Lamarkiana* having four bivalents and a ring of six chromosomes, found frequent interlocking of the bivalents both with the ring and with each other. He found that the relative chances of a bivalent interlocking with the ring or with another bivalent (3.13 : 1) are proportional to the relative lengths. The interlocking in this *Oenothera* appeared to occur at random between the bivalents. Like Darlington (9), Catcheside concludes that interlocking is a mechanical result of the pairing of the chromosomes commencing at the ends; a conclusion subsequently disproved by Marquardt (21, see later).

Cleland (6) and Catcheside (5) found two kinds of interlocked rings in *Oenothera*: two rings locked as links of a chain, and rings locked to form an apparent multiple chiasma. Interlocked links result when one chromosome passes between two others near their centromeres, chiasmata being formed on either side of the centromeres and distal to the interlock and subsequently terminalizing. An interlock of the apparent multiple chiasma type presumably results from one chromosome passing between two others in the distal region, with chiasmata formed on either side in that arm and subsequently terminalized. Without resolution of the chromatids, however, the true nature of this latter type is clearly open to question.

Marquardt (21) compared two varieties of *Oenothera*, *Oe. Hookeri* and *Oe. Hookeri flava sulfurea* (genetically *Oe. Hookeri*, but in *suaveolens* cytoplasm). In *Oe. Hookeri* at diakinesis, 99.08% of the bivalents were rings with two end-connections each, while in *Oe. Hookeri flava sulfurea* 5.86% were rods with only one end-connection. The difference between samples of 200 cells was more than seven times the standard error and hence significant. When compared with regard to interlocking, however, they were found to be the same (21.5 and 22.4% respectively). Marquardt suggested that this independence of interlocking and end-connection frequency might be due to interlocking occurring only in the central heterochromatic region of the chromosomes, while chiasmata are always formed distal to this region. Interlocked rings, which simulate multiple chiasmata, would, however, result only from interlocking having occurred in the euchromatic distal regions; although three of this type were observed by Catcheside (5) in 331 diakinesis nuclei, Marquardt failed to find any in his material.

Anderson and Sax (1) found that the percentage of interlocking varied greatly within species of *Tradescantia*, the variation between plants being from 0 to 27%. Finding no corresponding variation in chiasma frequency, although according to Sax and Anderson (31) "the correlation between chiasma frequency and the percentage of interlocking is positive", they state that this variation in the percentage of interlocking is due to minor environmental factors not affecting chiasma frequency. In *Eremurus spectabilis*, Upcott (32) found in different groups of cells a variation of from 0 to 15% of the cells containing interlocked bivalents. She suggests that such local variation in the amount of interlocking might be due to variations in the speed of early prophase stages: if rapid, the chances of homologues lying

together before pairing begins would be remote, and interlocking would be probable; if slow, on the other hand, the homologues would have time to approach each other before pairing actually starts, and the chances of interlocking occurring would be small. These two explanations of the similar variations in *Tradescantia* and *Eremurus* are compatible, since minor environmental factors, such as change in temperature, might affect the rate of prophase.

Interlocking and Chiasma Formation

That the configurations of interlocked bivalents afford evidence for distinguishing between the two main theories of chiasma formation has long been recognized.

Sax (29), quoting Gairdner and Darlington's (14) data, concluded that the great preponderance of proximal interlocking in *Campanula* argues in favour of the alternate opening out theory, or else shows that chiasmata pass off the ends of the chromosomes before metaphase.

In *Tradescantia*, Sax and Anderson (31) found that 99.4% of the interlocks were proximal, and a similar percentage was reported by Catcheside (5) in *Oenothera*. They considered this to be strong evidence in favour of the "classical" theory of chiasma formation. Later, however, they reconsidered the importance of this excess (1), pointing out that it may be due to the pairing being started at and restricted to the ends.

This possibility invalidates such evidence in favour of the "classical" theory. It should be stressed, however, that in forms such as *Tradescantia*, *Oenothera*, and *Campanula*, in which chiasmata are largely terminal by metaphase, a preponderance of proximal interlocking is obligatory.

Four cases of "double" or chiasma interlocking have been reported, where a loop in one bivalent is locked with two adjacent loops of another and around a chiasma between them. The first such case was reported by Mather (22) in *Lilium regale*, and one has since been seen by Upcott (32) in *Eremurus spectabilis*, by Beal (2) in *Lilium elegans*, and by Straub in *Gasteria strigosa* (See Oehlkers (26)).

These authors all agree that unless there had been two chiasmata in the centre, one of which had subsequently broken, the configuration could not have arisen by alternate opening out. Loss of a chiasma is unlikely unless interlocking causes chiasma breakage, since there is no reduction in the number of chiasmata from diplotene to metaphase in *Eremurus* and very little in *Lilium*. These materials, however, were mass stained and it would therefore be difficult, if not impossible, to distinguish between one chiasma and two very close together. Cases of chiasma interlocking are nevertheless strong evidence in favour of the partial chiasmatsby theory.

Mather (23) found no appreciable reduction in chiasma frequency in a limited number of interlocked bivalents in *Lilium* and claimed that this is to be expected on his assumption, since crossing-over would be unaffected by

interlocking at pachytene. Yet Gelei (15) and others have shown that the interlocked chromosomes interfere with the pairing of the interlocking chromosomes. Moreover, Dark (7) found only one chiasma in an interlocked bivalent instead of the two or three found in 48 other cases.

Gairdner and Darlington (14), Huskins and Smith (17), Dark (8), Upcott (32), and Darlington (12) all state that interlocked bivalent configurations were found which were explicable only on the partial chiasmata theory of chiasma formation, and not on the classical theory.

Interlocking and Terminalization of Chiasmata

Gairdner and Darlington (14) have stated that the occurrence of distal interlocking shows that terminalization had occurred without breaking of chiasmata; it cannot, therefore, be assumed that chiasmata ever break (or pass off the ends) during terminalization in the absence of this "exceptional strain" imposed by interlocked chromosomes. Sax and Anderson (31) obtained evidence from *Tradescantia* that interlocks are not released by chiasmata passing off the ends, and Marquardt (21) later showed that the frequency of end-connections in *Oenothera* is independent of the interlocking frequency.

According to Upcott (33) movement of chiasmata is slight in *Tulipa*. Where two bivalents each with one chiasma in each arm are interlocked, however, there is complete terminalization of the chiasmata, a condition never observed in free bivalents of this type. This throws further light on the mechanics of terminalization. Evidently the body repulsions of the chromosomes, increased by interlocking, reinforce their centric repulsions, and are strong enough to bring about terminalization of the chiasmata.

In *Pisum sativum*, Pellew and Sansome (27) found a configuration in which two pairs of segments in a segmental interchange ring of four were interlocked. The number of chiasmata was unusually high, 10 being observed, four more than had been observed in any other configuration. They suggest that interlocking might increase the number of chiasmata either by increasing the number of breaks in the chromatids at earlier stages or more probably by hindering terminalization. The former possibility is in disagreement with, among others, Dark's (7) observation on *Scilla* and Mather's (23) on *Lilium*. Since their configuration consisted of distal interlocking, and cancellation of interstitial chiasmata is apparently not proved, it may reasonably be assumed that distal interlocking would tend to hinder terminalization of the proximal chiasmata, whereas proximal interlocking would tend to aid in the terminalization of distal chiasmata.

General Appearance of Interlocked Bivalents

Mather (23) found in *Lilium* that when bivalents were interlocked the loops involved were generally enlarged. A similar condition was observed by Upcott (32) in *Eremurus spectabilis*. She also found that the two bivalents orientate themselves at right angles, as do successive loops of one bivalent, this obviously being the position of equilibrium. In the case of false interlocks both Mather

and Upcott state that the outer bivalent is always found to be around a chiasma in the inner one. Anywhere else the repulsion of the expanding arms of the encircled loop would tend to push the encircling loop toward one or the other chiasma. A single exception was found by Upcott, but the inner loop was not fully expanded. She suggests that in this case the outer bivalent must have been in the central equilibrium position, where the forces tending to push it towards one chiasma equalled those tending to push it towards the other. The appearance of these interlocked bivalents and the cases of terminalization of chiasmata noted by Upcott (33) in *Tulipa* offer further evidence of the non-specific nature of the repulsions between chromosomes.

As a result of interlocking the proper orientation of the bivalents on the metaphase plate is frequently upset (7, 8, 11, 12, 32).

Morgan, Bridges, and Sturtevant (25), Belling (4), and Sax (28) have suggested that interlocking may cause segmental interchange between non-homologous bivalents (translocation). Sax and Anderson (30) found an apparent correlation between interlocking and translocation in several species of *Tradescantia*, but admit that the interlocks might be the result rather than the cause. Finally, Koller (20) found in *Dasyurus* a configuration of two bivalents which indicated that they had been interlocked and then broken; the ends had rejoined in such a way that they would constitute a translocation.

Material and Methods

Rhizomes of *Trillium erectum* were collected at Ste. Agathe, Que., in September and October, 1937 and 1939. Slides were made from 10 rhizomes kept during meiosis in chambers maintained at various temperatures (Table I). At the appropriate time the anthers were removed, smeared, desiccated for

TABLE I
FREQUENCY OF INTERLOCKING

Slide	Temperature during meiosis	No. of cells examined	Cells with interlocks, %	Cells with true interlocks, %	Cells with false interlocks, %	Bivalents involved in interlocks, %	Interlocks*, %	
							True	False
66-T-89	3° - 4° C.	100	18	9	11	7.8	10	11
66-T-85	4° - 6° C.	200	33	18	17	14.2	19.5	20
66-T-81	8° - 10° C.	100	26	9	18	10.2	9	18
58-5d	16° C.	100	21	10	12	9.2	11	13
65-M-9	22° - 24° C.	200	56	39	31.5	29.4	54.5	43
Mean			34.7	20.3	19.7	16.3	25.4	24
58-2E	4° C.	113	3.5	1.8	1.8	1.5		
58-3D	8° C.	100	17	9	9	3.6		
58-4E	11° C.	100	6	1	5	2.4		
58-5G	16° C.	100	13	7	7	2.8		
58-6X	20° C.	100	6	3	3	2.4		

* See pp. 369 and 370.

20 to 30 sec., and fixed in La Cour's 2BD fixative for about two hours. The slides were then washed in water, bleached in a solution of equal parts of hydrogen peroxide and 70% ethyl alcohol for one or two hours, and stained with crystal violet according to the method described by Huskins and Smith (17).

The "58" slides were similarly prepared by Dr. G. B. Wilson and Slide 65-M-9 by Dr. H. B. Newcombe.

Observations were made with a Zeiss 1.5 mm., 1.3 N.A. objective combined with 7 \times , 15 \times , and 20 \times oculars. Drawings were made with the camera lucida at original magnifications of 3875 \times and 5000 \times . Those reproduced herein bear no constant relationship to these original magnifications.

Observations

Two distinct lots of slides were examined (Table I). Those in the first were especially chosen because of the high frequency with which interlocking was observed; the second lot was selected later and examined only in order to determine whether or not there was a relationship between interlocking frequency and the temperature at which meiosis had occurred—a relationship implied by Upcott (32) but not substantiated by our observations on the first lot. All drawings, measurements, and other detailed analyses have been made from the slides comprising the first lot; the second will be omitted from considerations apart from the effect of temperature (see later).

General Appearance of Interlocked Bivalents

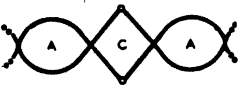
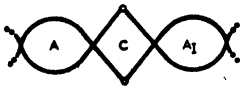
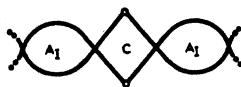
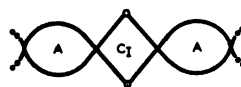
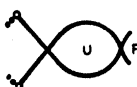
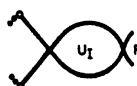
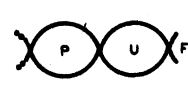
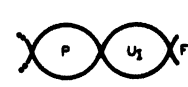
Early prophases were not examined for interlocking, but interlocked bivalents were observed at late diplotene, diakinesis, and metaphase. These interlocks were of two main types, termed in conformity with earlier workers, true interlocks (Fig. 1) and false interlocks (Fig. 2). Incomplete true interlocks were also found, in which one of the bivalents involved had failed to form the necessary distal chiasma. There is, therefore, the possibility that such interlocks might slip apart both before and during metaphase orientation of the bivalents on the equatorial plate. Likewise, since false interlocks are not held in place by chiasmata in both bivalents, it should be possible for them to slip apart before metaphase.

In the case of late diplotene interlocks the bivalents concerned show no definite orientation with regard to each other, frequently lying side by side with even the interlocked loops nearly parallel. By diakinesis, however, the bivalents generally lie at right angles to each other. In cases where the bodies of the bivalents lie nearly parallel, the interlocked loops, at least, tend to be almost at right angles to each other. This orientation of the loops appears to be an equilibrium position attained as a result of the mutual repulsion of the chromosomes.

In determining the effect of interlocking on loop size, comparisons have been made in two distinct categories. The first (Table II, 1 to 4) compares the size of the centric loop (C) with that of the loops (A) immediately adjacent to it and thus observations have necessarily been restricted to bivalents having

at least two chiasmata in each arm. The second (Table II, 5 to 8) compares the most distal, or ultimate loop (U) with the length of the free end (F) and,

TABLE II
EFFECT OF INTERLOCKING ON LOOP SIZE

Type of configuration	Relative size	Significance	Percentage length of bivalent involved
1 	a. $C > A = 33$ b. $C = A = 1$ c. $C < A = 62$	$\chi^2 = 8.8; P = < 0.01$	$C = 13.0$ $A = 15.5$
2 	a. $C > A = 7$ b. $C < A = 13$ c. $C > A1 = 3$ d. $C < A1 = 17$ e. $A > A1 = 6$ f. $A < A1 = 14$	$\chi^2 = 1.8; P = < 0.20$ $\chi^2 = 9.8; P = < 0.01$ $\chi^2 = 3.2; P = > 0.05$	$C = 14.1$ $A = 19.6$ $A1 = 25.9$
3 	a. $C > A1 = 0$ b. $C < A1 = 4$		$C = 11.1$ $A1 = 32.9$
4 	a. $C1 > A = 8$ b. $C1 < A = 0$		$C1 = 29.1$ $A = 15.6$
5 	a. $U > 2F = 5$ b. $U < 2F = 17$	$\chi^2 = 6.6; P = 0.01$	$U = 20.2$ $F = 23.6$ $\frac{1}{2}C = 6.2$
6 	a. $U1 > 2F = 21$ b. $U1 < 2F = 10$	$\chi^2 = 3.8; P = 0.05$	$U1 = 33.8$ $F = 9.3$ $\frac{1}{2}C = 7.0$
7 	a. $U > 2F = 3$ b. $U < 2F = 6$ c. $U > P = 5$ d. $U < P = 4$ e. $P > 2F = 4$ f. $P < 2F = 5$		$U = 10.8$ $P = 15.0$ $F = 16.8$ $\frac{1}{2}C \text{ or } R = 7.4$
8 	a. $U1 > 2F = 21$ b. $U1 < 2F = 0$ c. $U1 > P = 19$ d. $U1 < P = 2$ e. $P > 2F = 15$ f. $P < 2F = 6$	$\chi^2 = 3.86; P = < 0.05$	$U1 = 27.2$ $P = 13.2$ $F = 4.3$ $\frac{1}{2}C \text{ or } R = 5.3$

Note.—Mean percentage length of non-interlocked loops = 15.0.

Mean percentage length of interlocked loops = 29.5.

Mean of 2 + 3: $C = 13.6$.

$A1 = 27.1$.

in arms with three chiasmata, with the proximal or penultimate loop (P). The lengths were determined from camera lucida drawings and are expressed as percentages of the lengths of the chromosome.

In the absence of interlocking, C is somewhat smaller than A (Table II, 1). With an interlock in one of the A loops (A_1), that loop becomes larger than C in 17 out of the 20 cases measured (Table II, 2, c vs. d) while the other A loop, lacking an interlock, is larger only 13 times out of 20 (Table II, 2, a vs. b). The mean distance from the centromere to the chiasma on the interlocked side is 7.2, which is not significantly greater than the distance from the centromere to the chiasma on the non-interlocked side (6.9). The A_1 loops are also larger than the A loops in 14 out of the 20 cases. The mean percentage of C is considerably less than the mean of A_1 and, what is more remarkable, appreciably smaller than the mean of A . The C loop was found only twice with interlocks on both sides (Table II, 3); all these four A_1 loops were larger, with a mean of 32.9% against 11.1 for the C . It is worthy of note that the C mean, even with interlocks on both sides, is not appreciably smaller than when there is only one or none. Finally, on the eight occasions when the interlock involved the centric loop (C_1 , Table II, 4), C_1 was always larger than the A loop, the mean of A in this case being approximately the same as when the bivalent is free from interlocking.

In the second analysis the ultimate loop (U) in two-chiasma arms free from interlocking was found to be *smaller* than twice the free end ($2F$) on 17 occasions out of 22, while, with interlocking in the loop (U_1), it was *greater* on 21 out of 31 (Table II, 5 vs. 6). In three-chiasma arms without interlocking (Table II, 7) the mean lengths of the ultimate and penultimate loops (P) were both less than that of the free end. Finally, with interlocking in the ultimate loop, however, P remains about as before, F is reduced to about $\frac{1}{2}$, but U_1 is increased to about two and one-half times (Table II, 8).

It should be noted in this connection that loops containing more than one interlock are considerably larger than the adjacent non-interlocking loops

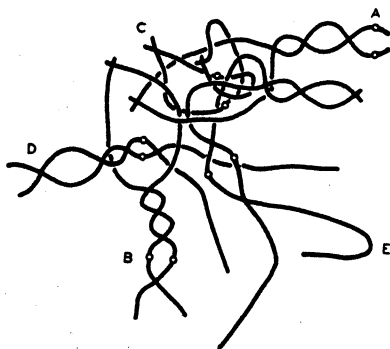


FIG. 3. Simplified drawing of the most complicated cell analysed. False interlocks:— A encircling C and E , B encircling D . True interlocks:— A and B , A and C , C and E twice.

(Fig. 3) and larger than loops containing only one (Fig. 5)—this is generally true.

Unlike the condition reported by Mather (23) in *Lilium*, and, with one exception, by Upcott (32) in *Eremurus*, where the outer bivalent of a false interlock is always around a chiasma in the inner one, in *Trillium* it is found that the outer bivalent is as frequently between chiasmata in the inner one as it is around a chiasma.

In cells containing interlocked bivalents the number of bivalents involved varied from one locked on itself to all five involved in interlocks. In Plant 65-M-9, where the frequency of interlocking was highest, one cell was seen with seven interlocks (Fig. 3), one with six, one with five, and five with four.

The three bivalents *C*, *D*, and *E*, with median or submedian centromeres (Fig. 4), were all found with their two arms locked together, the *E* 18 times, and the *C* and *D* five times each. The interlocks were true in 17 cases and false in 11. The subterminal *B* bivalent was also found locked on itself on one occasion, but the lock involved the long arm only; it was a true interlock.

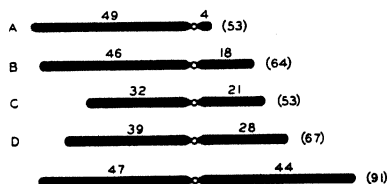


FIG. 4. The relative lengths of the chromosomes and chromosome arms.

Three instances were observed in which two bivalents were locked together in two separate places. In Plant 65-M-9, the *D* and *E* bivalents were locked in a true interlock, and one chromosome of the *D* also passed through another loop of the *E* in an incomplete true interlock. In another cell the *A* bivalent was locked around the *B*, and another loop of the *A* was also around one chromosome of the *B* forming an incomplete true interlock. In a third cell the *D* and *E* bivalents were doubly interlocked, there being a true interlock in both arms of each. In Fig. 3 the arms of the *C* have both formed true interlocks with one loop of the *E* and one is involved in a false and the other in a true interlock with one loop of the *A*. Finally, this same plant showed two cases in which the *E* bivalent was locked on itself and also with the *A*. In one, both the interlocks were true, in the other both were false. Three bivalents interlocked as links of a chain were found frequently in all plants except 66-T-81, in which only one out of the 100 cells analysed contained more than one interlock.

Only one case of chiasma interlocking occurred among the total of 346 interlocks found and this was in 65-M-9. One loop of the *B* bivalent was locked with two adjacent loops of the *D*, and around the chiasma between them (Fig. 5). It is noteworthy that this is the only case of chiasma interlocking so far reported in which the chromatids could be clearly traced. It

could be plainly seen that there was only one chiasma in the *D* bivalent between the pairs of chromatids of the *B*. The two arms of the *D* bivalent were also locked together in a true interlock, the loop in one arm being the same one as that locking the *B* chromosome.

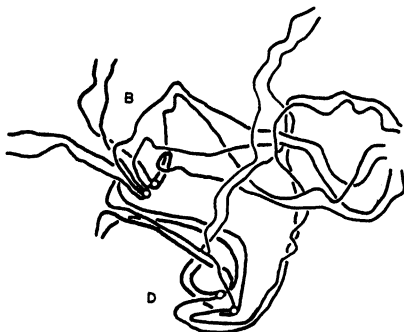


FIG. 5. The *B* bivalent doubly interlocked around a chiasma in the *D*,—the two arms of the *D* are locked together.

In 66-T-85 an example of chromatid interlocking was found (Fig. 6); in the loop next to the centric loop of the *E* bivalent one of its chromatids passed between the chromosomes of the *C*. If this chromatid had been outside the *C* instead of through it, the configuration would have been an ordinary false interlock of *E* around *C*. On the other hand, if the outer *E* chromatid had been alongside the inner one the configuration would have been a normal true interlock.

Frequency of Interlocking

Although interlocked bivalents have been seen in all *Trillium erectum* slides prepared in this laboratory, in the selected slides comprising the first of the

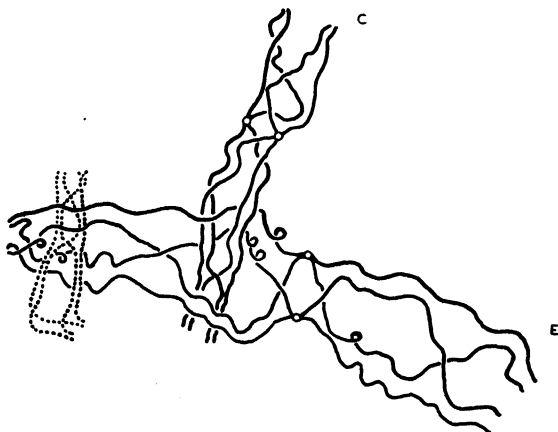


FIG. 6. The *C* bivalent locked around one chromatid of the *E* (semidiagrammatic).

two lots examined (Table I) the frequency of their interlocking was especially high. All percentages are based on counts of at least 100 cells—the actual numbers examined are given in Table I. The frequency of interlocking can be expressed in a number of ways. Here it is expressed as follows:— (1) the percentage of cells in which interlocking was observed, either true, false, or both combined, (2) the percentage of bivalents involved in interlocking, and (3) the number of interlocks, either true, false, or both, actually observed per 100 cells, i.e., the percentage of interlocking.

From 18 to 56% of the pollen mother cells in the first lot contained interlocked bivalents, the mean frequency being 34.7%. This is considerably higher than has been reported in most other materials, with the possible exception of ring-forming plants, such as *Oenothera*, in which 50% or more of the cells have been found to contain interlocks.

True interlocks were found in from 9 to 39% of the cells, the mean being 20.3%, while false interlocks varied in frequency from 11 to 31.5%, with a mean frequency of 19.7%. The ratio of cells containing true interlocks to those containing false interlocks (20.3 to 19.7) is approximately 1 : 1; the actual numbers of the two types of interlocking found (178 true and 168 false) are also approximately in the ratio of 1 : 1. The values of the true interlocks, both here and elsewhere, include, as well as ordinary true interlocks, any incomplete true interlocks found. These incomplete true interlocks constitute about 22.0% of the total number of true interlocks found.

The percentage of interlocks seen in the various plants ranged from 21 to 97.5 with a mean of 49.4%. In general, the percentages of true and false interlocks were not significantly different, but in plant 66-T-81 there were twice as many false as true. It is of interest to note that although plant 65-M-9 had interlocks in only 56% of the cells examined, when they are expressed as mean number of interlocks per cell, i.e., as percentage of interlocking, there is an average of almost one per cell. This is due to the relatively high frequency of cells containing more than one interlock found in this plant, as has been mentioned previously.

As shown in Table I the plants in the first lot underwent meiosis at temperatures ranging from 3° to 4° C. to 22° to 24° C. Plant 66-T-89, kept at 3° to 4° C., contained the least interlocking, regardless of how it is expressed, while plant 65-M-9, at 22° to 24° C., showed the highest value. These two values taken alone would seem to indicate a direct correlation between temperature and the amount of interlocking. Plants kept at intermediate temperatures, however, show a reverse relationship between temperature and interlocking. Plant 66-T-85, at 4° to 6° C., contained interlocked bivalents in 33% of the pollen mother cells, 66-T-81, at 8° to 10° C., in 26%, and 58-5d, at 16° C., in only 21%. It seems evident, therefore, that there is no simple correlation, either positive or negative, between the temperature at which the plants underwent meiosis and the amount of interlocking. To check whether or not the two opposite relationships shown were the result of some cause other

than direct temperature, the second lot of plants was studied. As is apparent from Table I, they showed that there is no direct relationship with temperature.

The respective frequencies with which the five bivalents were involved in interlocking were determined (Table III). These values were subdivided into four groups. The first, total interlocks, shows the frequency with which the bivalents were involved in interlocking of any kind. The second, true interlocks, shows the frequency with which they were involved in true interlocks. The third and fourth groups are composed of the bivalents involved in false interlocks, these being divided into the encircling and the encircled bivalents.

TABLE III
BIVALENTS INVOLVED IN INTERLOCKING

Slide	Total interlocks					True interlocks				
	A	B	C	D	E	A	B	C	D	E
66-T-81	12	10	1	8	23	4	3	0	2	9
66-T-85	35	33	9	23	58	19	15	4	10	30
66-T-89	6	8	2	9	17	2	6	1	4	7
65-M-9	77	71	52	64	126	38	45	29	37	69
58-5d	12	10	3	5	18	5	5	2	1	9
Total	142	132	67	109	242	68	74	36	54	124
Percentage	20.5	19.1	9.7	15.8	35.0	19.1	20.8	10.1	15.2	34.8

False interlocks

Slide	Encircling bivalents					Encircled bivalents				
	A	B	C	D	E	A	B	C	D	E
66-T-81	4	3	0	5	6	4	4	1	1	8
66-T-85	6	9	3	5	17	10	9	2	8	11
66-T-89	3	1	0	2	5	1	1	1	3	5
65-M-9	11	14	11	13	37	28	12	12	14	20
58-5d	4	2	0	1	6	3	3	1	3	3
Total	28	29	14	26	71	46	29	17	29	47
Percentage	16.7	17.3	8.3	15.5	42.3	27.4	17.3	10.1	17.3	28.0

Although the totals for true and false interlocks were almost the same, as were the relative frequencies of the five bivalents concerned in them, the relative frequencies of the encircling and encircled A and E bivalents in false interlocks were significantly different. The frequencies observed for individual plants are too low for their variations to have much significance, but in the case of the encircled bivalents of the false interlocks it is noteworthy that in Plant 65-M-9, which has the largest number of interlocks and in which variation should, therefore, be most significant, the value for the subterminal

A is 40% higher than that for the median *E*. Excluding Plant 65-*M*-9 from the totals for encircled bivalents the remaining values are *A*, 18 (22%), *B*, 17 (21%), *C*, 5 (6%), *D*, 15 (18%), and *E*, 27 (33%). These percentages correspond quite closely to those for the other groups, suggesting that some unusual condition, or conditions, in Plant 65-*M*-9 may be responsible for the difference between the encircled bivalent group and the encircling and true groups.

In an attempt to find whether this difference might not be due, in part at least, to special affinities between certain pairs of chromosomes, the false interlocks were analysed to determine the relationship between the encircling and the encircled bivalents (Table IV). Although the numbers are not very large it will be seen that the encircling *A* bivalents, whose frequency is only about half that of the encircled *A* bivalents, are encircling each of the different bivalents roughly half as often as the *A* bivalents are encircled by the corresponding pairs. Similarly the excess of encircling *E* bivalents is distributed among the other bivalents in a proportion fairly close to that in which the *E* bivalents are encircled by each of the other four bivalents. It therefore appears probable that the poor fit between the encircling and encircled bivalents is largely due to the *A* and *E* bivalents, which are inversely affected in the two categories. Further, the difference seems to lie mainly in Plant 65-*M*-9.

TABLE IV
FALSE INTERLOCKS

Encircled bivalents	Encircling bivalents					Total
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>E</i>	
<i>A</i>	0	12	5	7	22	46
<i>B</i>	6	0	3	4	16	29
<i>C</i>	5	5	1	0	6	17
<i>D</i>	3	3	2	2	19	29
<i>E</i>	14	9	3	13	8	47
Total	28	29	14	26	71	168

Bivalent Pairs

If Sax and Anderson (30) are correct in their suggestion that interlocking is the result of intertwining of the prophase threads owing to the interchange of very short chromosome segments, then it might be expected that certain bivalents would tend to be locked with each other in a disproportionately large number of cases.

In order to test this possibility the interlocks were analysed with respect to the pairs of bivalents involved (Table V). The false interlock values were taken regardless of which of the bivalents was the encircled and which the encircling one. The relative percentages of the 10 types of bivalent pairs

TABLE V
PAIRS OF BIVALENTS INVOLVED IN INTERLOCKING

Slide	A-B	A-C	A-D	A-E	B-C	B-D	B-E	C-D	C-E	D-E
66-T-81	4	0	2	6	0	1	5	0	1	5
66-T-85	9	2	6	18	1	6	17	0	6	11
66-T-89	0	1	0	5	1	2	5	0	0	7
65-M-9	22	9	11	34	8	13	25	6	19	26
58-5d	4	1	0	8	1	1	5	0	1	2
Total	39	13	19	71	11	23	57	6	27	51
Percentage	12.3	4.1	6.0	22.4	3.5	7.3	18.0	1.9	8.5	16.1
65-M-9										
Percentage	12.7	5.2	6.4	19.6	4.6	7.5	14.4	3.5	11.0	15.0

are markedly different. The *E* is involved in each of the three highest percentages and the *C* in the three lowest; since these are respectively long and short chromosomes it appears that a relationship exists between interlocking frequency and chromosome length. Such a relationship would be expected if interlocking is a chance phenomenon. The fact that not only all bivalents but all bivalent arms are involved, and then not always in the same positions, renders an explanation based on segmental interchange highly improbable.

The analysis was also carried out with the values for total interlocks broken down into true and false. The relative percentages of the 10 types of bivalent pairs are similar in true, false, and total interlocks, in spite of the very small numbers in certain categories. This similarity would seem to indicate that in this respect, unlike the individual bivalent values, the false interlocks behave in the same manner as the true ones. The cause of this similarity lies in the fact that the deficiency of encircling *A* bivalents relative to encircled *A* bivalents is compensated for by the deficiency of encircled *E*'s relative to encircling. In so cancelling each other out the total interlocks are similarly affected and the observed agreement between total, true, and false is, therefore, not unexpected.

Position of Interlocking

Earlier analyses of the position of interlocking have mainly been concerned with whether the interlock occurs in the centric loop ("proximal interlocking") or in some other loop ("distal interlocking"). As evidence for distinguishing between the classical and the partial chiasmata theories of chiasma formation the importance of the ratio of proximal to distal interlocking is slight, since it may be affected by other factors. More important is the consideration of whether interlocking occurs in odd- or even-numbered loops from the centromere.

In Table VI the position of interlocking is analysed. The loops are numbered outwards from the centromere, the centric loop being labelled 0, the

next loop 1, and so on, irrespective of which arms of the bivalents are involved. It should here be noted that since the centric loop can encircle a chromosome only so long as there is at least one chiasma in each arm and because the arms of the bivalents are scored separately, the frequency of centric interlocks given in Table VI is necessarily twice the true frequency.

TABLE VI
LOOPS INVOLVED IN INTERLOCKING

Plant	Number of chiasmata per arm																		
	1		2		3		4		5		1 to 6*								
	Loop involved																		
	0	0 1	0 1 2		0 1 2 3		0 1 2 3 4		0 1 2 3 4 5										
66-T-81	1	2 8	1 3 6		0 0 0 2		- - - - -		4 11 6 2 - -										
66-T-85	4	0 36	0 9 9		0 1 0 2		- - - - -		4 46 9 2 - -										
66-T-89	1	1 8	0 0 2		- - - - -		- - - - -		2 8 2 - - -										
58-Sd	3	1 3	2 6 5		2 0 0 1		- - - - -		8 9 5 1 - -										
65-M-9	10	2 36	5 22 10		3 11 2 5		1 2 1 1 3		22 71 14 6 3 -										
Total	19	6 91	8 40 32		5 12 2 10		1 2 1 1 3		40†145 37 11 3 0										

* 65-M-9 had two six-chiasma arms with the zero and second loops respectively involved.

† Note that the zero loop is actually involved only 20 times when the bivalent is considered as a whole.

Of the 215 interlocks analysed 156 occur in the odd loops where, without interstitial loss of chiasmata, interlocking is impossible according to the classical theory. On the partial chiasmotypy theory, the distribution of interlocks into odd and even loops should, other things being equal, be dependent on the chiasma frequency. Taking into account the disparity in numbers of odd and even loops in the arms with uneven numbers of chiasmata, we find that instead of the proportion of odd to even being about 89 : 126, as it should on a random basis, it is 156 : 59. Clearly some factor or factors are influencing the distribution of the interlocks; what these may be will be discussed later.

Interlocking and Chiasma Frequency

In view of the interference in pairing resulting from interlocking, observed by many authors at pachytene, and the general increase in size of the interlocking loops seen at later stages in the present material, the working hypothesis that interlocking causes a reduction in chiasma frequency was tested. If this were so a progressive reduction should occur in bivalents of the following types:— (1) encircled bivalents in false interlocked pairs, (2) non-interlocked bivalents, (3) true-interlocked bivalents, and (4) encircling bivalents in false interlocked pairs. The encircled bivalent type in false interlocks was tentatively expected to have a higher chiasma frequency than non-interlocked bivalent because, in order to be encircled, chromosomes of this type must have been closely associated at the time of pairing.

We have determined the chiasma frequencies of the five different bivalents, A to E, in all five plants, grouping the four plants with the lowest interlocking frequency, and treating 65-M-9 separately since it has sufficiently high numbers in all categories and, incidentally, has the highest chiasma frequency. In the case of the four plants the analysis has been carried out for cells having no interlocks and for individual bivalents involved in true interlocks and the two types in false interlocks.

The data for the first four plants are given in Table VII and expressed as the mean chiasma frequencies of the individual bivalents, with a grouped mean for the set of five bivalents in each of the four categories. There are thus six lots of comparisons possible between individual bivalents of the five

TABLE VII
EFFECT OF INTERLOCKING ON CHIASMA FREQUENCY

Plant			A	B	C	D	E	Mean chiasmata per five bivalents
66-T-85, 66-T-81, 66-T-89, and 58-5d	False interlocks	No. chiasmata observed	19	30	15	21	45	19.41
	Encircled bivalents	No. bivalents observed	7	8	4	5	9	
		Mean chiasmata per bivalent	2.71	3.75	3.75	4.20	5.00	
65-M-9	Cells without interlocks	"	64 25 2.56	102 25 4.08	74 25 2.96	93 25 3.72	124 25 4.96	18.28
	True-interlocked bivalents	"	49 19 2.58	55 18 3.06	21 6 3.50	40 10 4.00	135 28 4.82	17.96
	False interlocks	"	29 11 2.64	25 7 3.57	8 3 2.67	31 7 4.43	55 12 4.58	17.89
	Encircling bivalents							
	False interlocks	No. chiasmata observed	40	35	22	16	45	24.30
	Encircled bivalents	No. bivalents observed	11	6	5	4	7	
		Mean chiasmata per bivalent	3.64	5.83	4.40	4.00	6.43	
	Cells without interlocks	"	105 24 4.38	104 24 4.33	76 24 3.17	120 24 5.00	147 24 6.12	23.00
	Non-interlocked bivalents in cells with interlocks	"	110 30 3.67	110 27 4.07	129 39 3.31	149 32 4.66	108 17 6.35	22.06
	True-interlocked bivalents	"	45 14 3.21	56 14 4.00	35 9 3.89	54 11 4.91	128 22 5.81	21.82
	False interlocks	"	10 4 2.50	22 6 3.67	17 5 3.40	22 5 4.40	62 9 6.89	20.46
	Encircling bivalents							

types, or a total of 30. When the comparisons are made according to the above hypothesis, it is found that observation fits expectation on 20 occasions, or in 67% of the cases. Further, it will be noticed that when the means are determined for sets of five bivalents, expectation is fulfilled in all four categories.

Similar data are also given in Table VII for Plant 65-M-9 with the addition of the mean chiasma frequencies of non-interlocked bivalents occurring in cells having interlocked bivalents. This analysis has been made to ascertain whether the conditions that reduce the number of chiasmata in interlocked bivalents also extend their influence to the accompanying non-interlocked bivalents. In the latter, potential interlocks may not materialize owing to a failure to form the necessary chiasmata. Here there are 50 possible comparisons of which 33, or 66%, were found to fit the hypothesis. Moreover, the mean chiasma frequency per set of five bivalents in the five categories shows the trend expected.

Discussion and Conclusions

Most of the data presented may be divided into two classes, (1) those concerned with the mechanics of interlocking, how interlocking occurs, and the forces governing the behaviour of interlocked bivalents, and (2) those that provide evidence concerning the method of pairing and chiasma formation. There is, of course, some overlapping between those two classes, since certain data have a bearing on both, but as far as possible they will be discussed separately.

Mechanics of Interlocking

The very variety of types of interlocking found indicates a certain randomness in their formation. All combinations of pairs of bivalents were found, true and false occurred in almost equal numbers, the two arms of bivalents with submedian centromeres were locked together, and the subterminal *B* was locked on itself. With the exception of the *B* bivalent, the remaining self-locked bivalents, 28 in all, were locked across the centric region, suggesting that the centromere is a point of prophase polarization. The observation of statistical equality of true and false interlocks (actually a slight excess of the latter) effectively refutes for *Trillium* both Dark's (8) suggestion that false interlocks may be due to postzygotene movement and Darlington's (9) and Upcott's (32) contention that false interlocks slip apart by metaphase.

(a) Bivalent Ratios

If interlocking is purely a matter of the chance relationship of the chromosomes to each other at the time of zygotene pairing, it seems reasonable to suppose that the ratio of the frequencies with which the five bivalents are involved in interlocks would be directly proportional to the total lengths of the chromosomes. To test this possibility the frequencies which would be expected for each bivalent were calculated, the lengths used being those shown in Fig. 4. These lengths were those determined by Huskins and Smith (17)

from measurements made at pollen grain mitotic anaphase. Since only relative lengths and not absolute lengths were needed for these calculations it was thought that they would be sufficiently accurate. In the case of the *A* bivalent the length used was that of the long arm only, since the centromere is so nearly terminal that it may be considered to be so in effect. The calculated values were compared with the values actually found (Table III), and χ^2 tests made to determine whether or not the values found fitted the hypothesis (Table VIII). The tests were made separately for the total interlocks, true interlocks, and the two types of bivalents in false interlocking.

As may be seen from Table VIII the value of χ^2 varied from 52.09 for the total down to 21.61 for the encircling bivalents of the false interlocks. With four degrees of freedom these all give probability values of considerably less than 0.01. It is obvious from this that the relative frequencies of the bivalents involved in interlocking do not fit the hypothesis.

TABLE VIII
BIVALENT RATIOS IN RELATION TO CHROMOSOME LENGTH

	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>E</i>	χ^2	P ($N = 4$)
Total interlocks							
No. observed	142	132	67	109	242		
No. expected	104.7	136.7	113.2	143.1	194.4	52.086	<0.01
True interlocks							
No. observed	68	74	36	54	124		
No. expected	53.8	70.3	58.2	73.6	100.0	23.391	<0.01
False interlocks							
Encircling bivalents							
No. observed	28	29	14	26	71		
No. expected	25.4	33.2	27.5	34.7	47.2	21.606	<0.01
Encircled bivalents							
No. observed	46	29	17	29	47		
No. expected	25.4	33.2	27.5	34.7	47.2	22.184	<0.01

It was noticed that in the 132 cases in which the *B* bivalents were involved in interlocking the short arm was involved only once. This suggested the possibility that a minimum length of chromosome arm might be necessary for interlocking. If this were so it seemed reasonable to suppose that the short arm of the *B* must approximate very closely to this minimum length. On this assumption it is not the total length of the chromosomes which should be used to calculate the expected frequency of each bivalent, but the total length with the minimum value, 18, subtracted from each arm. Therefore, instead of 49, 64, 53, 67, and 91 for the *A*, *B*, *C*, *D*, and *E* bivalents respectively, 31, 28, 17, 31, and 55 should be used. In Table IX the frequency found for each bivalent is compared with the expected frequency calculated from the corrected lengths above.

The χ^2 values for the total interlocks, true interlocks, and encircling bivalents of the false interlocks on this basis were 6.76, 5.61, and 5.92 respectively.

TABLE IX

BIVALENT RATIO IN RELATION TO CHROMOSOME ARM LENGTHS MINUS A MINIMUM VALUE

	A	B	C	D	E	χ^2	P ($N = 4$)
Total interlocks							
No. observed	142	132	67	109	242		
No. expected	132.4	119.6	72.6	132.4	234.9	6.765	<0.10
True interlocks							
No. observed	68	74	36	54	124		
No. expected	68.1	61.5	37.4	68.1	120.9	5.609	<0.20
False interlocks							
Encircling bivalents							
No. observed	28	29	14	26	71		
No. expected	32.2	29.0	17.6	32.2	57.0	5.916	<0.20
Encircled bivalents							
No. observed	46	29	17	29	47		
No. expected	32.2	29.0	17.6	32.2	57.0	8.006	<0.05
Encircled bivalents excluding 65-M-9							
No. observed	18	17	5	15	27		
No. expected	15.7	14.2	8.6	15.7	27.9	2.101	>0.70

With four degrees of freedom these give values for P of less than 0.10, 0.20, and 0.20 respectively. These three types, therefore, all fit the minimum value hypothesis. In the case of the encircled bivalents of the false interlocks, however, χ^2 was 8.01, giving a P value of less than 0.05. It is due to the exceptionally high value for the A bivalent and the low value for the E , that this category does not fit the hypothesis as closely as the others do, but following the use of the corrected lengths there is a very substantial reduction in the χ^2 value, which is comparable to the reduction in the other three categories. It will be remembered that the encircled A bivalents of the false interlocks in Plant 65-M-9 occurred with a frequency 40% greater than that of the encircled E bivalents. When the 65-M-9 values for all five bivalents are omitted from the present analysis, the remainder fit the minimum value of hypothesis closely with a χ^2 of 2.10, giving $P = 0.70$.

From the results of the above tests it may be stated that, except possibly in the case of the encircled bivalents of false interlocks in 65-M-9, the frequency with which bivalents are involved in interlocks is proportional to the relative lengths of their arms minus a minimum length equal to that of the short arm of the B chromosome. This minimum length may be necessary for the formation of an interlock either because pairing is initiated at or near the centromeres, which are probably polarized (see later), or because it is the shortest length that will permit the formation of an interlock without inhibiting the formation of the distal chiasma necessary for its realization.

(b) Bivalent Pairs

Having considered the bivalents individually the next consideration is pairs of interlocked bivalents. Do the 10 types of bivalent pairs occur in the ratio expected from the frequency of interlocking of the individual biva-

lents? This would indicate randomness. Or do certain pairs occur with a frequency significantly greater than expected? This would indicate some specific attraction between the pairs such as might result from prior segmental interchange between them.

TABLE X
INTERLOCKING FREQUENCY OF PAIRS OF BIVALENTS

	A-B	A-C	A-D	A-E	B-C	B-D	B-E	C-D	C-E	D-E
Five plants										
Total observed	39	13	19	71	11	23	57	6	27	51
Total expected	37.83	16.58	28.81	59.94	15.18	26.37	54.87	11.56	24.06	41.79
	$\chi^2 = 12.92, N = 9, P = 0.20$									
True observed	21	3	9	35	3	16	32	4	18	19
True expected	19.91	7.74	13.27	28.76	8.20	14.05	30.45	5.46	11.84	20.30
	$\chi^2 = 13.02, N = 9, P = <0.20$									
False observed	18	10	10	36	8	7	25	2	9	32
False expected	17.75	8.88	15.61	31.22	6.96	12.23	24.47	6.12	12.24	21.52
	$\chi^2 = 15.75, N = 9, P = >0.10$									
65-M-9										
Total observed	22	9	11	34	8	13	25	6	19	26
Total expected	15.97	11.70	14.39	28.33	10.78	13.27	26.13	9.72	19.13	23.55
	$\chi^2 = 7.28, N = 9, P = 0.60$									

To answer these questions the frequencies expected for the various bivalent pairs were calculated from the frequencies of the individual bivalents excluding those locked on themselves. In Table X the frequencies found, separated into total, true, and false interlocks, are compared with the expected values, calculated from the frequency of interlocking of the individual bivalents as stated above. These three comparisons give χ^2 values of 12.92, 13.02, 15.17, which, with nine degrees of freedom, give values for P of 0.20, <0.20 , and >0.10 . Clearly, the frequencies observed correspond closely to the calculated ones. Since the objection might legitimately be raised that the use of the grouped totals from five unrelated plants would tend to minimize any inherent differences, the analysis was carried out separately for total interlocks in 65-M-9, the plant with the greatest incidence of interlocking and thus the one in which the test should be most significant. As is shown in Table X the value for χ^2 was 7.28, giving P equal to 0.60. It may be concluded from this that no specific attraction exists between any of the pairs of bivalents, the interlocking of these pairs being entirely random. This evidence is contrary to Sax and Anderson's proposal that interlocking may be the result of very short exchanged segments of chromosomes that cause the attraction and consequent interlocking of otherwise non-homologous chromosome pairs. It also renders improbable Dark's (8) suggestion that in tetraploids interlocking

is due to a failure to form the chiasmata necessary for quadrivalent formation among homologues closely associated either by secondary or pachytene pairing.

(c) *Position of Interlocking*

Interlocking was found in every internode of the bivalents except the fifth—there were, however, only two arms with as many as six chiasmata. The interlocks were particularly frequent in the proximal, i.e., the loop next to the centric loop, and the most distal loops, and occurred in them in about equal numbers. When these two coincide, i.e., in two-chiasma arms, the ratio of centric to proximal was 1 : 15, far lower than in any other case (Table VI).

If interlocking is a random phenomenon, the chance of an interlock arising in any particular loop should be dependent solely on the mean length of the loop. The one loop which is fixed in its position is, of course, the centric loop. Its mean length has been determined from measurements of 86 bivalents, each devoid of interlocking, and expressed as a percentage of the length of bivalent lying between the most distal chiasmata of the arms. The percentage of this length occupied by the centric loop was found to be 26.8 and on the assumption of randomness 26.8% of the interlocks in interlocked bivalents should therefore occur in the centric loop. Considering only bivalents with chiasmata in each arm the percentage of centric interlocks is, however, only 9.3 or one-third of the expected value (Table VI). This deficiency of centric interlocks would be expected if pairing were initiated at the centromeres and the proximal parts of the chromosomes were in an orderly spatial arrangement; it certainly does not indicate a fortuitous distribution at leptotene. Nor is it in agreement with the conclusion of many workers (5, 9, 24), that pairing starts at the distal ends.

(d) *Orientation of Interlocked Bivalents*

At late diplotene interlocked bivalents were found to take up no definite position with regard to each other. By late diakinesis or metaphase, however, they tend to be at right angles to one another as was noted by Upcott (32). This observation is in agreement with the generally accepted concept that by diakinesis there is developed a repulsion not only between the chromosomes of a bivalent but also between non-homologous chromosomes. It may reasonably be supposed that these are not two separate forces, but one non-specific repulsion. In the case of false interlocking bivalents both Mather (23) and Upcott (32) report that, with one exception noted by the latter, the encircling bivalent was always around a chiasma in the encircled bivalent. They hold that this is a natural result of the general repulsion operating between non-homologous bivalents. The absence of such a constant relationship in *Trillium erectum* is evidence that the force of repulsion in it is relatively weak, as is further instanced by the stable position of chiasmata, the rarity of terminalized chiasmata even when distal to an interlock, and the relative closeness of bivalents in general.

(e) *Interlocking and Loop Size*

It was noticed that the loops involved in interlocking were larger than the comparable ones free from interlocking on 69 occasions and smaller on only 14. The enlarged interlocking loops suggest two possibilities. The first, that the presence of another chromosome between a pair of homologous chromosomes may prevent the formation of a chiasma close to it; the second, that the development of the body repulsions of the chromosomes may cause the interlocking loops to become enlarged by the movement of chiasmata away from the interlock. Either of these possibilities might also explain the minimum length found necessary for interlocking. According to the first a subterminal interlock would be prevented by the inhibition of the distal chiasma necessary for its retention. According to the second the chiasma distal to the interlock might be forced off the end of the bivalent, thus allowing the two bivalents to slip apart.

That neither of these explanations is entirely adequate is shown by the following considerations. First, even in those bivalents having at least one chiasma in each arm, the frequency of centric interlocking is only about one-third of that expected. Second, the majority of interlocks occur in the most distal loop, where, if expansion caused by interlocking were sufficient to force a distal chiasma off, their frequency should be lowered. Admittedly, the penultimate loop would then assume the distal position and its interlocks would themselves become distal, but this would necessitate a direct correlation between interlocking and chiasma frequencies whereas the reverse has been found here. Further, it would demand a decreasing order of interlocking in the loops from the most distal to the most proximal, in order to fit the observation that, regardless of the stage of meiosis, the most distal loop has a high, if not the highest, frequency of interlocking.

A detailed analysis of Table II allows a choice between these two possibilities. With regard to the possible effect of repulsion on loop size, it is clear that if there is an effective repulsion the loop size can be changed by one or both of two movements—a shift of the proximal chiasma towards the centromere and a shift of the distal chiasma towards the end. That there is no movement *towards* the centromere in *Trillium* is clearly shown by Comparisons 1, 2, and 3 in Table II, where the *C* loop is of uniform size regardless of whether interlocks occur in one, both, or neither of the adjacent loops. This is shown even more clearly by the fact that in Category 2 the mean distance from the centromere to the proximal chiasma does not differ significantly in the presence or absence of an interlock (7.2 vs. 6.9 respectively). However, when an interlock occurs in the adjacent loop the overall length of the centric and adjacent loops is significantly greater than when there is none. Thus, grouping interlocks in Categories 2 and 3, we have a percentage length of 40.7 against 28.5 in Category 1. Since the centric loops are approximately equal, the size difference must be due to the relative positions of the distal chiasmata in the two cases. So far there is no conclusive evidence whether this is the result of simple interference with chiasma formation, or

of increased repulsion which is effective in moving chiasmata distally but ineffective in moving them proximally.

The fourth analysis supplies more definite evidence. In this case the proximal chiasmata are displaced distally by the presence of an interlock in the centric loop, leaving the centric loop about twice the size it is in the former analyses. Furthermore, the second chiasma completing the adjacent loop, is also displaced distally and displaced to such an extent that the loop, despite the "infringement" by the proximal chiasma, is nevertheless as large as when no interlock is present. This suggests that the increased size of interlocked loops is the result of simple mechanical interference and has no direct influence on the size of adjacent loops. This is illustrated beyond reasonable doubt in Table II where both $C + A_1$ and $C_1 + A$ occupy approximately 40% of the bivalent. Since the displacement due to interlocking occurs distally and not proximally, pairing in *T. erectum* must initially proceed from the centromere outwards along the arms.

Analyses 5 to 8 in Table II are in complete agreement with this conclusion and afford additional evidence on the time sequence of pairing. In two-chiasma arms the increase in size of loops involved in interlocking is gained at the expense of the distal portion, i.e., the free ends; thus $U + F = 20.2 + 23.6 = 43.8$ and $U_1 + F = 33.8 + 9.3 = 43.1$. The remaining part of the arm, R or $\frac{1}{2}C$, is therefore unaltered (6.2 vs. 7.0). Hence we conclude that in two-chiasma arms, or, on the average, in shorter arms, pairing starts at the centromere and moves outwards *to the ends*. Arms with three or more chiasmata are more variable distally and, consequently, the derived values will be less significant. One fact is established, however, and that is that little of the excess size of interlocked ultimate loops is gained at the expense of the more proximal loops; thus in 7 and 8, $P = 15.0$ and 13.2 respectively. It is gained largely from the free ends for their mean percentage lengths are 4.3 with interlocking and 16.8 without interlocking, a difference of 12.5 compared with a difference between the loops of 16.4. Part of the inequality is doubtless due to the variation in chiasma frequency in the arms comprising these categories, for this will cause differences in the length of bivalent occupied by the three distal chiasmata and the free end.

Frankel (13) has suggested that in the long arms of *Fritillaria* chromosomes there is, in addition to the primary centric pairing, a secondary pairing point located at the end of the chromosome. There appears to be no clear evidence of any such subsidiary pairing point being consistently operative here. Mather (23) has expressed the opinion that interlocking at pachytene would have no effect on chiasma frequency; this is contrary to the results obtained from the above analysis. Because a larger part of the bivalent is occupied by interlocking loops as compared with non-interlocking loops there should be a reduction in the chiasma frequency of interlocked bivalents (see later).

(f) *Randomness of Bivalents Involved in Interlocks*

The observations here made on *Trillium erectum* support the hypothesis, proposed by many workers, that bivalent interlocking results from a mechanical accident occurring during zygotene pairing. When homologous chromosomes pair one or both chromosomes of another pair may be caught between them. With the formation of the necessary chiasmata, these interlocks are retained as true and false interlocks, respectively. This theory would also explain the equal frequency of true and false interlocking, the random interlocking of the five bivalents in relation to the relative lengths of their arms minus a minimum length, and the random associations of pairs of bivalents in the ratio expected from their individual frequencies.

The exceptional behaviour of the *A* and *E* bivalents comprising the false interlocks in Plant 65-*M*-9 requires further consideration. Based on the grouped frequency with which the various bivalents were involved in true interlocks, there was a 34% deficiency of encircled *E* bivalents and a 24% excess of encircling *E* bivalents; in the case of the *A* there was a 70% excess of encircled bivalents and a 33% deficiency of encircling bivalents. Evidently some factor was acting to increase the chance of the *E* encircling another bivalent and to decrease its chance of being encircled. This factor, or a second one, was affecting the *A* bivalent in the reverse manner. Since this factor (or factors) must be operative at the time of pairing, it appears probable that the underlying cause may be a difference in linear homology resulting in the *E* chromosomes remaining apart longer and hence rendering them more liable to encircle a second bivalent. More or less complete homology of the *A* chromosomes would ensure their more immediate association, thus increasing their chance of encirclement by another bivalent and reducing the probability of their encircling another. In Plant 65-*M*-9 some evidence for a lack of linear homology was observed at anaphase in the form of dicentric chromatids, but unfortunately it was not possible to identify the bivalent involved.

Chiasma Formation

(a) *Position of Interlocking*

Interlocking was found in both odd and even loops, in every loop, in fact, apart from the fifth, from the centric loop to the most distal (Table VI). According to the classical theory of chiasma formation it should occur only in the even loops, except for occasional cases where a chiasma had broken. It was found, however, that, taking into account the disproportion of odd to even loops, the odd loops were interlocked three times as often as the even loops. This evidence is thus directly contrary to the classical theory.

Mather (23) has postulated that the position of interlocking is dependent on the method of pairing; that with pairing starting at the ends interlocking would be most frequent in the centric loop, while randomness in pairing would be associated with randomness in their spatial distribution. Apparently he follows Catcheside (5) and Darlington (9) in assuming that pairing progression ("zipper pairing") is uninterrupted by interlocked chromosomes which instead

are pushed towards the centromere. From the present study it appears, on the contrary, that the distribution of interlocks is dependent primarily on the spatial relationship of the threads at the time of pairing, and secondarily on the position in which chiasmata are subsequently formed.

(b) *Interlocking and Chiasma Frequency*

That a correlation exists between interlocking and chiasma frequency has been suggested by various workers (27, 7, 31), but has been denied by others (23, 1). The metaphase chiasma frequency could presumably be affected by interlocking in two ways, the one acting *after* chiasma formation to reduce the number originally formed, the other acting *before* either to decrease or to increase the number subsequently formed. The first possibility would necessitate the dissolution of terminal chiasmata, or the cancellation of adjacent interstitial free compensating pairs and elimination of one in adjacent interstitial continuous non-compensating pairs. Dissolution of terminal chiasmata appears to be ruled out by Marquardt's (21) demonstration that the frequency of end-unions in *Oenothera* is independent of the interlocking frequency. Cancellation of interstitial free pairs and elimination of one in continuous pairs remains a possibility, but would require an average reduction per interlocked bivalent of one and one-half chiasmata, since free and continuous pairs occur in approximate equality (16). Apart from Pellew and Sansome's (27) observation of an exceptionally high chiasma frequency in a self-interlocked ring of four in *Pisum sativum*, there appears to be no unequivocal evidence for the existence of a positive correlation such as would result from interlocking determining an increase in chromatid breakage (chiasma formation) at pachytene. Evidence for a negative correlation, however, is observational—the interlocked chromosome or chromosomes interfere mechanically with the association of the encircling pair at pachytene.

The hypothesis that interlocking causes a reduction in chiasma frequency has been established by the analysis of four plants combined and Plant 65-M-9 separately (Table VII). The five bivalents, A to E, in the five categories analysed in 65-M-9: (1) encircled bivalents in false interlocks, (2) bivalents in cells free from interlocking, (3) bivalents free from interlocking in cells having interlocked bivalents, (4) bivalents involved in true interlocks, and (5) encircling bivalents in false interlocks, have mean chiasma frequencies which when summated for each category are 24.30, 23.00, 22.06, 21.82, and 20.46, respectively. The question now arises, is the progressive reduction due to, (a) loss of terminal chiasmata which allows the slipping apart of interlocked bivalents, (b) cancellation or elimination of interstitial chiasmata, or (c) simple mechanical interference with the association of chromosomes at pachytene?

The first possibility can be dismissed immediately since the lowest chiasma frequency is not associated with the lowest frequency of interlocking. The second, cancellation and elimination of adjacent interstitial chiasmata, requires the opposite relationship, i.e., the relationship observed. As already mentioned, since free pairs constitute approximately half the chiasma

pairs formed and continuous pairs the majority of the remainder, this second possibility requires a reduction in chiasma frequency of 1.5 per bivalent (in bivalents with four or more chiasmata, Table XI) or 6.0 per set of five

TABLE XI

INTERLOCKING AND THE CANCELLATION OR ELIMINATION OF ADJACENT INTERSTITIAL CHIASMATA

Chromosome	Initial chiasma frequency (approx.)	Reduction with adjacent pair free	Reduction with adjacent pair continuous	Resulting chiasma frequency
<i>C</i>	3	0	0	3.0
<i>A</i>	4	2	1	2.5
<i>B</i>	4	2	1	2.5
<i>D</i>	5	2	1	3.5
<i>E</i>	6	2	1	4.5
Total	22	8	4	16.0

bivalents. The maximum difference observed, that between encircling and encircled bivalents of false interlocks, was only 3.84. If, on the other hand, the increased size of interlocked loops were the result of interference, the reduction in chiasma frequency would be proportional to the increase in loop size. Taking the increase in loop size as the mean of centric, adjacent, penultimate, and ultimate interlocked loops minus the mean for the corresponding loops free from interlocking, there is a total reduction of 14.5% in the length of bivalent remaining in which chiasmata can be formed. There should, therefore, be a similar percentage reduction in chiasma frequency between non-interlocked and interlocked bivalents. The mean chiasma frequency for non-interlocked bivalents in non-interlocked cells was 23.00 while the mean frequency for true and encircling bivalents was 21.14, giving an observed percentage reduction of 8.1. Now since at pachytene non-interlocked bivalents may have been potential interlocks that failed to materialize due to a failure to form the chiasmata necessary for their retention (see Categories 2 and 3 above), it may perhaps be justifiable to calculate the reduction on the basis of encircled rather than non-interlocking bivalents (for the reason given earlier). When this is done the observed percentage reduction in chiasma frequency, 13.0, fits expectation, 14.5, closely. We, therefore, conclude that the reduction in chiasma frequency is the result of interference rather than the cancellation and elimination of interstitial chiasmata.

(c) Chiasma Interlocking

The one case of chiasma interlocking found in Plant 65-M-9 is the first reported where the chromatids can be traced (Fig. 5). It is perfectly clear that only one chiasma is present in the *D* bivalent between the chromosomes of the *B*. Interpreted on the classical theory this would mean that sister chromatids were separated, passing on either side of one chromosome of the *B*,

in one loop, or that there had been two chiasmata in the *D* bivalent between the chromosomes of the *B*, one of these chiasmata subsequently having broken. The explanation on the partial chiasmotypy theory is far simpler and involves no abnormal condition; sister chromatids are paired in both the loops of the *D* bivalent concerned, and the chiasma arose as a concomitant of crossing over.

(d) Chromatid Interlocking

One case of chromatid interlocking was observed in Plant 66-T-85 (Fig. 6). Since all the chromatids could be traced there was no doubt that the *C* bivalent was locked around a single chromatid of the *E*. Gairdner and Darlington (14) state that chromatid interlocking should occur if the chromatids ever separate equationally. However, neither theory of chiasma formation, as then formulated, will explain the configuration seen. It could, of course, result from accidental breakage on either theory. If crossing over is conditioned by torsion, in the way suggested by Darlington (10, 12, p. 483), the explanation of the interlocking of a single chromatid would be the breaking and untwisting of one of the chromatids of the *E* (separated by the *C*) and its reunion with one of the chromatids of the homologue to form a chiasma on the other side of the *C* chromosome. The fact that the interlock as observed is some distance from the chiasma may be due to repulsion having shifted the *C* bivalent proximally relative to the *E* bivalent.

The evidence obtained from this study of interlocked bivalents in *Trillium erectum* strongly supports the partial chiasmotypy theory of chiasma formation, while no unequivocal evidence was found in favour of the classical theory.

Chromosome Orientation and Progression in Pairing

Based on the observation of (1) the relative frequency of interlocking between the two arms of a bivalent as opposed to interlocking within an arm, (2) the occurrence of centric interlocks with a frequency only one-third of that expected from the mean length of the non-interlocked centric loops, and (3) a minimum length determining the length-frequency relationship in all five bivalents, it is concluded that the centromeres are polarized, and that the proximal regions of homologues are in an orderly spatial arrangement. The centromeres, then, have an initial advantage in pairing. Being on the average more closely associated than are other regions of the chromosomes, the proximal parts will be first aligned for zygotene pairing, which will then progress, necessarily outwards, towards the ends of the arms. If, at the same time, other homologous regions lie sufficiently near one another (such as the ends, as a concomitant of centric polarization) pairing will be independently started and will progress, presumably, both proximally and distally. Non-homologous segments and interlocked chromosomes will, of course, cause a cessation of the centrically initiated progression. This may be overcome by two means, the one dependent on distal association of homologous parts, the other being a restoration of the earlier progression by relational twisting carrying the association across the non-homologous region of interference.

Unpublished data on wheat (18) show that both of these may fail. Chromosomes heterozygous for an interstitial deficiency fail in bivalent formation more often than when homozygous for the deficiency. Since the pairable length of chromosome is the same in both, the difference in bivalent frequency must result from the presence in the heteromorphic pair of the univalent segment. Further, although the frequency of bivalent failure is proportional to the length of the heterozygous deficiency, there is no corresponding failure when the deficiency is homozygous. Clearly, there would be no such lack of agreement if pairing started at both the centromere and the ends; Darlington's (12, p. 166) contention, based on Kihara and Nishiyama's (19) work on wheat hybrids, that pairing in *Triticum* starts at the ends is invalidated on other grounds by Huskins and Smith (18) and by Huskins, Sander, and Love (manuscript in preparation).

From the analysis of interlocking in relation to loop size it has been seen that an interlock has little or no influence on the position in which the proximal chiasma is formed, its main effect being to shift the distal chiasma towards the end. This may be interpreted as favouring relational coiling rather than distal contact as the means of re-establishing association, or that progression in pairing is slower towards the centromere than away from it. Finally, the fact that only one chiasma interlock was observed among the 346 interlocks found is strong evidence for polarization and also for mechanical interference consequent on interlocking.

Further evidence based on a study of somatic pairing in a dipterous parasite relates this phenomenon to polarization and the pairing of chromosomes at meiosis (Smith, in press).

Acknowledgments

The authors wish to express their gratitude to Professor C. L. Huskins and Dr. G. F. Sander for reading and criticizing the manuscript, and to Dr. H. B. Newcombe and Dr. G. B. Wilson for use of certain of their slides.

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A NEW HEMIASCOMYCETE¹

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Abstract

A new genus and species are established for a Hemiascomycete growing on *Corticium microsporum*, a member of the Telephoraceae. The fungus is known from three collections made in different localities in Ontario. It is characterized by a limited mycelial growth in the subhymenium of the Basidiomycete where it also produces more or less elongate and coiled ascogonial branches and hypha-like antheridial branches, each female branch producing a single ascus which grows through the hymenium of the supporting fungus and protrudes prominently beyond the basidia. Phylogeny in the lower Ascomycetes is considered briefly and evidence is presented indicating relationship of the fungus in question with *Dipodascus* and *Endomyces*.

Introduction

In most classifications of the Ascomycetes there appears under the name used in the title above, a variously delimited and more or less controversial group of genera or families of morphologically simple forms. In its narrowest sense, i.e., in the sense of Varitchak (4), who is supported by Nannfeldt (3), it embraces only the genera *Dipodascus* and *Ascoidea*; in the broader sense of Gäumann (2, Hemiascomycetes, p. 137-165), it encompasses all those Ascomycetes, including the yeasts and the Taphrinales, that lack an ascocarp. The group in either sense, but more especially with respect to the genera *Dipodascus* and *Ascoidea*, has a peculiar interest and importance to all students of phylogeny in the fungi because of the prominent position accorded its members in any consideration of the origin of the Ascomycetes. Historically it consists of a miscellaneous accumulation of doubtful, misunderstood, and unrelated species; but the research and thought of the past 10 years have contributed much toward a better knowledge of some of the species and toward a better understanding of the group as a whole. Evidence is accumulating (1, 3, 5, 6) which tends to knit the Hemiascomycetes, even in the broader sense of the term, more firmly together than was formerly suspected. Attempts at a natural classification, however, still are hampered by a scarcity of connecting forms and by a paucity of detailed knowledge of certain known species, so that the discovery and assiduous study of additional forms is greatly to be desired. It follows then that the finding of a new non-ascocarpous form possessed of a combination of characters which is rather profoundly at variance with any hitherto known for the group—and which for this reason may in the future contribute toward an understanding of the group as a whole—warrants a more extended consideration than might be justified were it merely an addition to the number of known fungi.

¹ Manuscript received May 14, 1942.

Contribution from the Laboratories of Cryptogamic Botany and the Farlow Herbarium, Harvard University, Cambridge, Mass., No. 210.

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The fungus under consideration produces asci in the hymenium of *Corticium microsporium* (Karst.) Bourd. & Galz., where they occur scattered among the basidia, are much larger than the basidia, protrude prominently, and unless observed with extreme care would be interpreted as cystidia of the host, thus causing the latter to be referred to *Peniophora*. The ascomycetous nature of these cystidium-like bodies and the true identity of the supporting fungus were recognized recently by Dr. H. S. Jackson of the University of Toronto, while reworking a collection which had been taken in Ontario in 1937, and a portion of the material was communicated to the writer. The same thelephoraceous species was again found by Dr. Jackson to be bearing the Ascomycete in two collections made by him in Ontario, one in the summer of 1939 and the other in 1941.

Since Ascomycetes of which the morphology is of such a nature as to give any suggestion at all of identity with this one are so few in number of species, are so seldom encountered in nature, and have been given such prominence in the literature, it seems unlikely that the one at hand could previously have been placed on record and now overlooked. As it appears to be undescribed, and further since there is no known genus in which it may be placed, it becomes necessary to establish both genus and species for it.

Description

Helicogonium gen. nov. Ascocarpio nullo; mycelio vegetativo parco, hyphis angustis, ramosis, septatis, gerentibus et antheridia et acrocarpia (ramos ascogoniiferos); ramo femina laxe convoluto, septato, ad apicem cellulam tumidam ascogonialem gerente; ascogoniis singulis singularem ascum efformantibus; ascis elongatis, sessilibus, cum ascogonio continuis, apice incrassato, obtuso et, cum sporae deliberantur, dissoluto; ascosporis exiguis, hyalinis, bicellularibus, octo, at dein formantibus sporas secundarias numerosas intra ascum.

Ascocarp lacking; vegetative mycelium scanty, the hyphae narrow, branched, septate, producing both antheridial and ascogonial branches; antheridial branch consisting only of a septate hypha; female branch loosely coiled, septate, bearing a swollen ascogonial cell at its apex; ascogonia each extruding a single ascus; asci elongate, sessile, continuous with ascogonium, the apex thickened, obtuse, dissolving to liberate the spores; ascospores hyaline, two-celled, originally eight, budding within the ascus.

Helicogonium Jacksonii sp. nov. Ascocarpio nullo; fungo consistente e mycelio vegetativo crescente in subhymenio hospitii sui et producente ramos sexuales mares et feminas in eo his producentibus ascis; mycelio vegetativo parco, hyphis hyalinis, flexuosis, ramosis, septatis, tenui tunicatis, 1.8–2.5 μ in diam.; ramo mare consistente ex hypha tenuicula septata, haud manifeste distincta ab hyphis vegetativis, apice perficiente fusionem cum cellula ascogoniali; ramo femina brevi, laxe convoluto, incrassato ad apicem vel prope apicem, efformantem regionem ascogonialem, quae mox separatur septo; cellula ascogoniali subincrassata, curvata, ascum singularem ad latus convexum

efformante; ascis initio forma papillarum latarum gaudentibus, maturitate elongatis, forma nonnihil variabili, late clavatis, subcylindraceis vel subfusiformibus, $28-45 \times 7.5-10 \mu$, subconstrictis ubi asci in ascogonia transeunt, sed ab his haud separatim dissepimento, apice obtuso-rotundato, $3-4 \mu$ crasso, apparenter demum destructo actione solvente, incolorabili iodi ope; ascosporis hyalinis, oblongo-fusiformibus, $7-11 \times 2.5-3 \mu$, bicellularibus, bi-seriatim vel multi-seriatim dispositis, gemmantibus dum inasco iam remaneant saepeque ad ultimam consumptis productione sporarum secundariarum; sporis secundariis formatis e sporis primariis gemmantibus ad apices suos, demum replentibus ascum, ellipsoideo-cylindraceis, $3-4.5 \times 1.5-2 \mu$, duas guttulas olei minutas includentibus. Canada: Provincia Ontario. Ad *Corticium microsporum* . . . ad lignum acerosum.

Ascogonium lacking; fungus consisting of vegetative mycelium growing in the subhymenium of its host and producing there both male and female sex branches, the latter giving rise to asci; vegetative mycelium scanty, the hyphae hyaline, flexuous, branched, septate, thin-walled, $1.8-2.5 \mu$ in diameter; male branch consisting of a slender, septate hypha not noticeably differentiated from the vegetative hyphae, fusing at its apex with an ascogonial cell; female branch short, loosely coiled, enlarged at or near the apex into an ascogonial region which is soon abjoined by a septum; ascogonial cell somewhat enlarged, curved, extruding a single ascus from the convex side; asci beginning as a broad papilla, elongating, at maturity somewhat variable in shape, broadly clavate, subcylindric, or subfusoid, $28-45 \times 7.5-10 \mu$, slightly constricted at juncture with ascogonium but not separated from it by a cross wall, the apex obtuse, rounded, $3-4 \mu$ thick, finally disappearing through solvent action, not coloured by iodine; ascospores hyaline, oblong-fusoid, $7-11 \times 2.5-3 \mu$, two-celled, biseriate or multiseriate or irregularly arranged, budding while still in the ascus and often entirely consumed in the production of secondary spores; secondary spores formed by budding from the ends of the primary ones, finally filling the ascus, ellipsoid-cylindric, $3-4.5 \times 1.5-2 \mu$, each containing two minute oil drops.

Canada: Province of Ontario: On *Corticium microsporum* (Karst.) Bourd. & Galz. on coniferous wood. Bear Island, Lake Temagami. Aug. 3, 1937. A. J. Skolko. Type. (In Farlow Herbarium and in University of Toronto Cryptogamic Herbarium No. 12919.)—On *Corticium microsporum* on wood of *Pinus strobus* L., Petawawa Forest Reserve, Chalk River, Ont. Sept. 10, 1939. H. S. Jackson. (In Farlow Herbarium and in University of Toronto Cryptogamic Herbarium, 17569.)—On *Corticium microsporum* on log of *Tsuga canadensis* (L.) Carr. Woods south of Aurora. Oct. 13, 1941. H. S. Jackson. (University of Toronto No. 17572, but entire specimen deposited in Farlow Herbarium.)

There is no obvious evidence, either macroscopic or microscopic, that the Ascomycete is injurious to the *Corticium* upon which it grows. Superficially the hymenial surface of the supporting fungus appears normal, and under the microscope the asci are seen among the normally sporulating basidia. The

Corticium is delicate and fragile with a rather compact hymenial region supported by a broad substratal zone of erect, branching hyphae, very loosely arranged. The ascomycetous hyphae appear to be confined to the subhymenial layer of the *Corticium* and to be so scanty that they cause no noticeable increase in the compactness of the tissue of that region. Also the hyphae are so similar to the basidiomycetous hyphae among which they occur that it is difficult to trace them, and the exact relationship of the *Helicogonium* to the fungus upon which it grows has therefore not been determined. Very suggestive, however, are the short, slender lateral branches (Figs. 12, 14) which are seen frequently in crushed mounts; these seem to have a slightly specialized apex about which torn wall tissue adheres loosely, and it is possible that food and anchorage is obtained through the actual penetration of these apices into the basidiomycetous hyphae. It is certain that the ascomycetous hyphae do not reach the substratum of the supporting fungus and also that they do not even extend into the broad open substratal zone of the latter.

The fungus exhibits considerable simplicity, the scanty vegetative mycelium producing only antheridial and ascogonial branches (see illustrations), the latter each producing a single ascus. There is no ascocarp; ascogenous hyphae are lacking; and there is no evidence of a conidial stage. The antheridial branch is scarcely or not at all distinguishable from the vegetative mycelium except as it is later detectable by its apical union with the female branch, which appears as an elongate lateral appendage (Figs. 1, 2) that becomes approximately once loosely coiled and develops a terminal, or subterminal, thickened ascogonial region. An ascogonial cell (Figs. 3 to 7, etc.) is abjoined by the appearance of a septum at the stalk end of the thickened portion. A septum forms at the opposite end, at the point of union with the antheridium, following copulation. The whole process, in so far as can be determined from the dried material studied, is reminiscent of that illustrated for *Endomyces Magnusii* Ludw. (2, p. 144). In no case, however, has it been possible to trace the two copulatory branches to the same hypha.

All figures from type material mounted in potassium hydroxide-phloxine; drawn at a magnification of 1835 and reduced in reproduction to 1180.

FIGS. 1, 2. Hyphae with coiled, unfertilized female branches.

FIGS. 3 to 9, 11, 12. Female branches to which male or antheridial branches have become attached.

FIGS. 10, 13. Immature asci.

FIG. 14. A more nearly mature ascus in which spore formation is in progress.

FIGS. 15, 22. Asci containing eight primary spores.

FIGS. 16, 17. Asci in which some of the eight primary spores have disappeared in the production of secondary ones.

FIG. 18. Ascus filled with secondary spores, the primary one having been entirely utilised in their production.

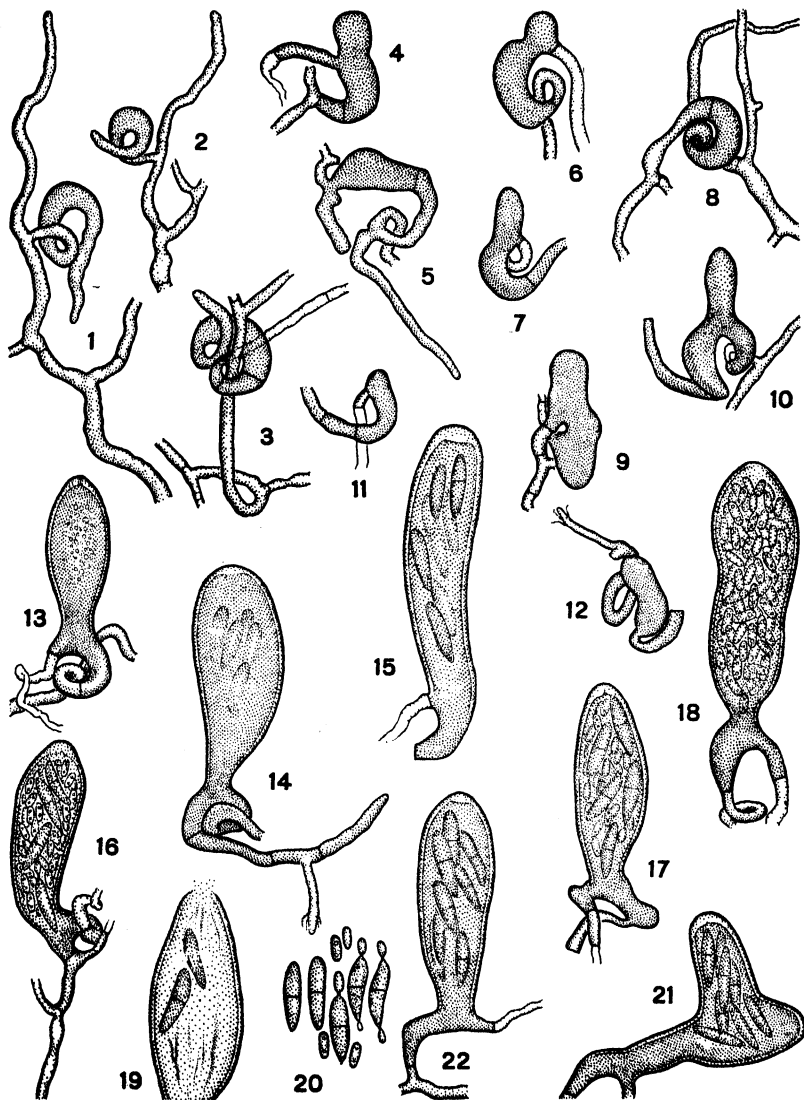
FIG. 19. Ascus at time of spore discharge showing free passage at apex formed by dissolution of the wall.

FIG. 20. Primary and secondary ascospores.

FIG. 21. An abnormal ascus.

For more detailed explanation, see text.

On the convex side of the inflated and somewhat curved ascogonium, and at a point furthest from the substratum, a rather broad and obtuse (Figs. 4, 5) bulge appears and elongates to form the single ascus (Fig. 10), which is merely a protrusion of the ascogonium. As the ascus elongates it broadens toward the apex (Fig. 13) so that at maturity there usually appears a slight constriction (Figs. 16, 18) at its juncture with the ascogonium, but there is no semblance of a stalk or pedicel and in no case is it cut off by a septum. The



ascus finally attains a size of $28-45 \times 7.5-10 \mu$, and its shape (Figs. 16 to 19, 22) is in general cylindric though it varies to broadly clavate or more rarely subfusoid. As the ascus develops (Figs. 13, 14) the apex thickens (Figs. 14 to 17), especially just before or at spore formation, and becomes broad and rounded; later it gelatinizes to form a free passage (Fig. 19) for the spores. At no time is it coloured by iodine. Occasionally there appears to be some evidence of a pore; at least if an ascus is crushed before gelatinization of the apex begins, the contents are forced out through a pore-like opening, and also a suture in the wall of the apex (Figs. 16, 17) is very frequently in evidence. This thickening probably is to be compared with that described and illustrated by Biggs (1) for *Dipodascus uninucleatus*, rather than with that exhibited by the asci of the higher, inoperculate Discomycetes.

Spore formation, in so far as can be determined from material mounted in potassium hydroxide-phloxine, appears to be exactly as it is in the higher Ascomycetes, and there is no reason to suspect that it is different in any way. Eight elongate spores (Fig. 20) are formed in each ascus (Figs. 15, 22) and they mature basipetalously (Fig. 14). Almost immediately a septum appears in each spore, dividing it into two equal parts, and this is followed by the formation of secondary spores (Fig. 20) that are budded off from the original eight spores until they fill the ascus and leave the primary ones entirely exhausted (Fig. 18). Only rarely are asci found (Figs. 15, 21, 22) in which the eight original spores are present.

Phylogenetic Considerations

If one were to claim a derivation of *Helicogonium* from somewhere among the higher Ascomycetes he might find certain characters to support his contention: coiled ascogonium, reduced male element, elongate and cylindric type of ascus, elongate and septate spores with the usual number of eight per ascus before budding, and the character of the ascus tip which may be interpreted as not unlike that to be expected from retrogression in the typical explosive tip. However, there appears to be no strong evidence for the derivation of any of the non-ascocarpous forms from ancestors that produced a fruit body; the gap between forms having an ascocarp with its usually accompanying ascogenous mechanism and the simplicity of a form like that at present under consideration is too complete to permit the assumption of such retrogressive phyletic transition.

More generally accepted and more easily supported is the evolutionary series recently set forth in some detail by Nannfeldt (3) and fairly well embodied by him in a taxonomic outline. It presupposes *Spermophthora Gossypii* to be the nearest present-day representative of the antecedent stock which gave rise to three developmental series leading into the present higher Ascomycetes, and a fourth series—"absteigende"—leading through *Dipodascus*, *Ascoidea*, *Endomyces*, and finally to the yeasts. There is no claim that the series is monophyletic; and whether or not its prehistoric antecedents corresponded very closely with the present *Spermophthora* is not pertinent to the

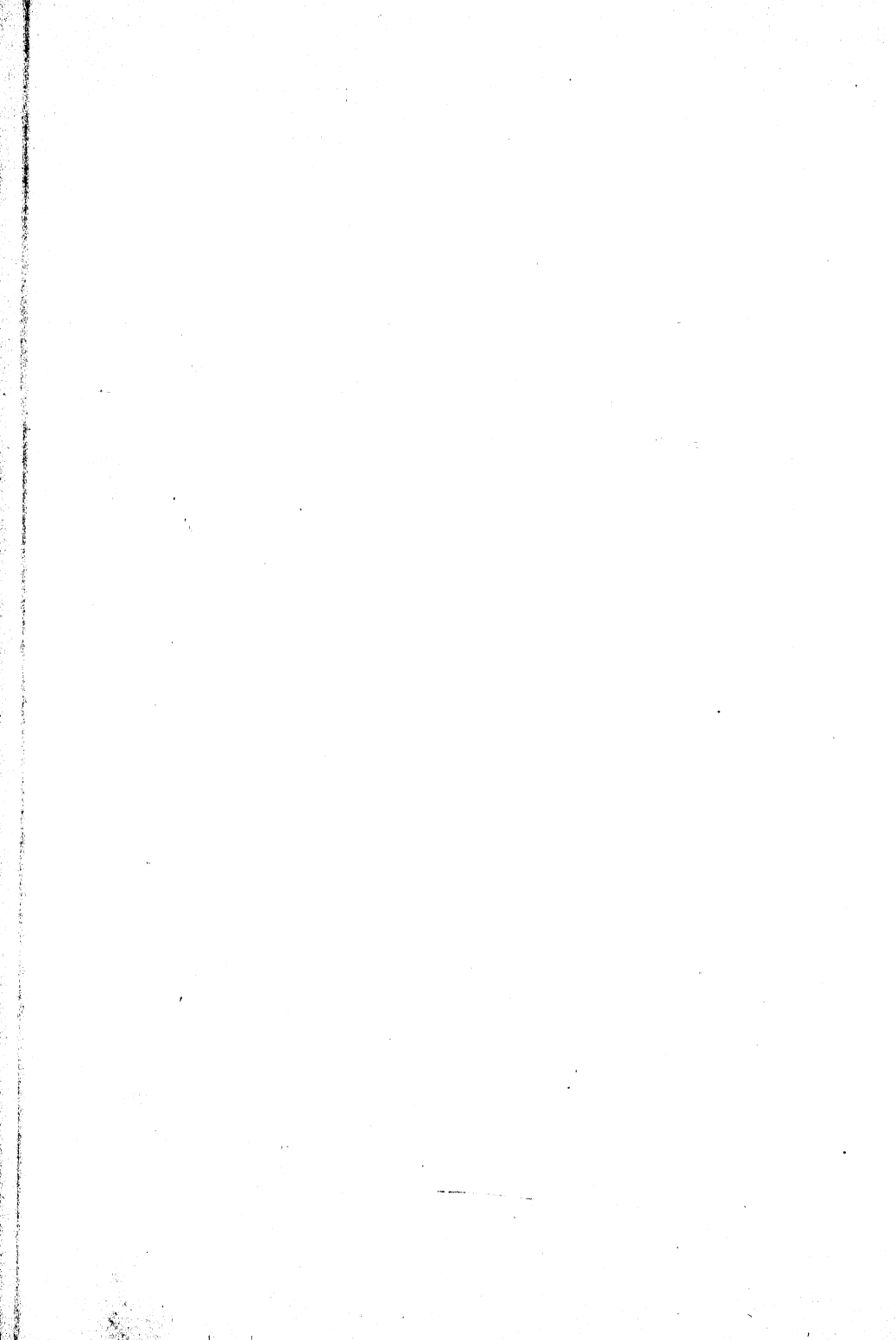
correct placement of the fungus at hand. Because it lacks a fruit body or an organized hymenium and because of the immediate production of an ascus following plasmogamy—other characters not prohibiting—it may for taxonomic purposes at least, be placed in the aforementioned series of Nannfeldt nearest the forms to which it shows the greatest morphological likeness—perhaps near *Dipodascus uninucleatus* Biggs or *Endomyces Magnusii* Ludw.

Acknowledgments

The author is indebted to his colleagues, Dr. D. H. Linder, Dr. E. V. Seeler, and Dr. Rolf Singer for assistance rendered in the course of the preparation of this paper, and to Dr. H. S. Jackson of the University of Toronto for having made available the material on which the study was based.

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RESEARCHES ON DROUGHT RESISTANCE IN SPRING WHEAT

II. THE EFFECT OF TIME OF DAY ON SURVIVAL OF PLANTS DURING EXPOSURE TO ARTIFICIAL DROUGHT¹

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Abstract

The time of day during which tests are conducted has a pronounced effect on the damage sustained by wheat plants exposed to artificial drought. When the day is divided into four six-hour periods, beginning at 6.00 a.m., the period from 6.00 a.m. to 12.00 noon provides the most severe test. In 12-hr. and 18-hr. exposures, relatively severe damage to the plants is associated with the inclusion of the 6.00 a.m. to 12.00 noon period.

Introduction

During the past five years, numerous tests of wheat varieties and strains have been conducted in the artificial drought machine described by Kenway and Peto (1). The results of preliminary trials revealed relatively enormous differences between the survival of experimental plants from one replicate to another. Since the machine had a capacity of only 25 6-in. pots, these differences had an important bearing on the design of experiments involving large numbers of varieties and strains. It was found that the time of day during which plants were exposed to artificial drought had a direct bearing upon the result. The purpose of this paper is to summarize data that serve to illustrate the relation between survival in the artificial drought machine and the time of day during which tests are conducted.

Peto (3) concluded, from experiments conducted in 1936, that wheat plants suffered relatively severe injury when the period extending from 6.00 a.m. to 12.00 noon was included in the test. He found that a 12-hr. exposure, beginning at 12.00 noon, effected approximately the same damage as a six-hour exposure beginning at 6.00 a.m. Laude (2) has obtained similar results with wheat, barley, corn, and sorghum. He states that "... the daily

¹ Manuscript received May 20, 1942.

Contribution from the Department of Field Crops, University of Alberta, Edmonton, Alta., with financial assistance from the National Research Council of Canada.

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maximum resistance to heat was attained by plants at about midday and continued during the afternoon. The minimum resistance prevailed early in the morning."

Materials and Methods

The material consisted of a number of varieties and strains of *Triticum vulgare* and two varieties of *T. durum*. In Experiment 1, there were 22 varieties, in Experiments 2 and 5 there were 25, while Experiments 3, 4, and 6 included 75.

The plants were subjected to treatment about a week before heading, though different varieties were, of necessity, at different stages of development. They were grown in ordinary 6 in. gardener's pots with drainage holes in the bottoms. The pots were watered before and after treatment by placing them in shallow trays which were filled with water. Since the porosity and, hence, evaporation rate varied from one pot to another, the pots were painted on the outside with asphaltum. In the early experiments, five plants were grown in each pot; but this number was reduced to four in later experiments.

The temperature in the machine varied not more than one degree above or below 110° F. The relative humidity varied from 14 to 23% from one experiment to another, but not more than 3% within six-hour tests, 5% within 12-hr. tests, and 7% within 18-hr. tests. The velocity of the air current was maintained at approximately 10 miles per hour.

The survival indices were based on the estimated percentage of uninjured tissue per pot. At first an attempt was made to classify each plant; but, finally, it was concluded that more consistent results could be secured by placing the pots on a bench in order of the severity of damage, and assigning a percentage survival figure to each.

Experimental Results

The results are assembled in Table I. The four periods of the day are designated as follows:

- A — 6 a.m. to 12 noon.
- B — 12 noon to 6 p.m.
- C — 6 p.m. to 12 midnight.
- D — 12 midnight to 6 a.m.

Each entry in the table, except the mean totals, represents the total survival percentages of all varieties in the test concerned. Thus in the six-hour exposures of Experiment 1 (22 varieties), the average percentage survival in the A period is 368/22, or 16.7%. In the D period it is 958/22, or 43.5%. In experiments involving 25, or 22, varieties, each figure is the result of a single "run", the capacity of the machine being 25 pots. Experiments 3, 4, and 6 each included 75 varieties and, consequently, three runs are involved in each entry.

In designing Experiment 1, no provision was made for separating the variance due to periods from that due to replicates, the reason being that the

• TABLE I

TOTAL SURVIVAL VALUES AND MEAN TOTALS OF ALL VARIETIES TESTED IN DIFFERENT PERIODS OF THE DAY

	Six-hour exposures			
	A 6 a.m. – 12 noon	B 12 noon – 6 p.m.	C 6 p.m. – 12 midnight	D 12 midnight – 6 a.m.
Experiment 1. 22 varieties; August 4 and 5, 1936	368 —	712 642	801 572	958 —
Mean totals	368	677	686	958
Experiment 2. 25 varieties; November 3 to 12, 1936	900 458 959	1625 — —	1757 — —	1166 — —
Mean totals	772	1625	1757	1166
Experiment 3. 75 varieties; February 16 to 21, 1937	1692 1123	1828 2918	1609 2272	— —
Mean totals	1408	2373	1940	—
Experiment 4. 75 varieties; May 9 to 14, 1937	3980 4750	5480 6000	5070 5620	— —
Mean totals	4365	5740	5345	—
	12-hr. exposures			
	AB 6 a.m. – 6 p.m.	BC 12 noon – 12 midnight	CD 6 p.m. – 6 a.m.	DA 12 midnight – 12 noon
Experiment 1. 22 varieties; August 5 to 8, 1936	— — —	325 308 317	— — —	75 112 104
Mean totals	—	317	—	97
Experiment 2. 25 varieties; November 3 to 12, 1936	122 —	756 883	156 —	343 526
Mean totals	122	820	156	434
Experiment 5. 25 varieties; May 17 to 22, 1937	1300 1530	1380 1650	1270 1770	940 1360
Mean totals	1415	1515	1520	1150

TABLE I—*Concluded*TOTAL SURVIVAL VALUES AND MEAN TOTALS OF ALL VARIETIES TESTED IN DIFFERENT PERIODS OF THE DAY—*Concluded*

	18-hr. exposures			
	<i>ABC</i> 6 a.m. – 12 midnight	<i>BCD</i> 12 noon – 6 a.m.	<i>CDA</i> 6 p.m. – 12 noon	<i>DAB</i> 12 midnight – 6 p.m.
Experiment 1. 22 varieties; August 8 to 13, 1936	0 —	312 —	319 195	0 0
Mean totals	0	312	257	0
Experiment 2. 25 varieties; November 3 to 12, 1936	116 — —	390 250 512	269 — —	251 — —
Mean totals	116	384	269	251

	Four-hour exposures		
	$\frac{1}{2}A$ 6 a.m. – 10 a.m.	$\frac{1}{2}A + \frac{1}{2}B$ 10 a.m. – 2 p.m.	$\frac{1}{2}B$ 2 p.m. – 6 p.m.
Experiment 6. 75 varieties; November 25 to December 1, 1937	1860 1260	3190 2950	3690 3780
Mean totals	1560	3070	3735

chief purpose was to classify varieties and strains on the basis of resistance to treatment, and the differential effect of periods on survival was not fully appreciated until this experiment was completed. The data derived from 12-hr. exposures, however, were adequate for statistical analysis, since there were three replicates in each of the *BC* and *DA* periods, and none in the other two. It is apparent (Table II) that the difference between the mean survivals of the *BC* (12 noon to 12 midnight) and *DA* (12 midnight to 12 noon) periods is highly significant.

Experiment 2, involving 6-, 12- and 18-hr. exposures, was designed so that the *A* period would be included in one-half of the replicates and not included in the other half. In the analyses (Table II), therefore, one degree of freedom was involved in the variance for exposure periods. In the six-hour exposures, the *A* period was much more damaging to the plants than were the others. The results of the 12-hr. exposures are not fully in accord with expectation (note Experiment 5), since the *CD* period was more damaging than *DA*. The results of the 18-hr. exposures suggest that the *A* period is more damaging when it occurs at the beginning of a run (*ABC*) than when it is in

the middle (*DAB*) or end (*CDA*). In general, the results of Experiment 2 lend weight to the belief that plants tested in the morning suffer greater damage than when tested during other periods of the day or night.

TABLE II
SUMMARY OF ANALYSES OF VARIANCE FOR EXPERIMENTS 1 TO 6

	Experiment 1		Experiment 2			
	D.f.	Variance (12 hr.)	D.f.	Variance (6 hr.)	Variance (12 hr.)	Variance (18 hr.)
Varieties	21	206.1	24	1939.8	765.4	294.3
Exposure periods	1	3290.0	1	33172.4	4309.4	1775.0
Varieties \times exposure periods	21	92.0	24	311.2	203.0	105.7
Replicates	4	10.2	4	3426.6	3834.0	483.6
Replicates \times periods	84	85.1	96	292.2	202.1	181.0
Residual						
Total	131		149			

	Experiment 3		Experiment 4		Experiment 5		Experiment 6	
	D.f.	Variance	D.f.	Variance	D.f.	Variance	D.f.	Variance
Varieties	74	443.9	74	516.7	24	1109.4	74	1106.3
Exposure periods	2	6237.0	2	13364.6	3	2409.3	2	33124.5
Varieties \times exposure periods	148	392.3	148	298.9	72	245.1	148	285.5
Replicates	3	3336.2	3	2590.6	4	2761.0	3	946.0
Replicates \times periods	222	285.2	222	343.4	96	221.4	222	205.9
Residual								
Total	449		449		199			

In experiments 3 to 6, the number of replicates was the same for each period involved. Experiments 3 and 4 are identical, except for the time of year, and the results agree in all essentials. The greater damage, in general, suffered by the plants in Experiment 3 is undoubtedly a result of the fact that they were less sturdy than those in Experiment 4. Differences in light conditions in the greenhouse between February and May were largely responsible.

The results of Experiment 5 are in conformity with the view that the *A* period is most damaging, but at variance with those of Experiment 2 (12- and 18-hr.) in that *DA* is more harmful than *AB*. The relatively small effect of the *A* period in Experiments 4 and 5 may well be due to the early hour of sunrise during the month of May, which tends to shift the *A* period towards *D*.

Experiment 6 provides results of considerable interest, and warrants the speculation that were the day to be further subdivided, the early part of the *A* period would be found to cause greater damage than the later part.

It is apparent from the results of statistical analyses recorded in Table II that observed differences between exposure periods are highly significant.

Differences in the reactions of varieties are clearly established in Experiments 2, 5, and 6, and suggested in the others. There was, however, little evidence of consistency in varietal reactions from one experiment to another. Under these circumstances, it is not surprising that no relation between the results of artificial drought tests and yield under field conditions could be established.

Discussion

That the time of day during which wheat plants are exposed to artificial drought has an important bearing on the results is established beyond a doubt. The marked susceptibility to treatment during the morning, and the development of resistance later in the day remains unexplained. There can be little doubt that light conditions are responsible for the differences observed; but discussion of the physiological processes involved cannot, at present, be justified.

Notwithstanding the varied response of different varieties to treatment, it is unlikely that the machine will be of use in selecting for resistance to drought. The results have failed to show any clear relation with yield under field conditions.

Acknowledgments

The authors are indebted to Dr. C. H. Goulden, Dominion Rust Research Laboratory, Winnipeg, and to Dr. J. W. Hopkins, Division of Biology and Agriculture, National Research Council, Ottawa, for advice in connection with experimental design and the statistical analysis of the results.

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POLARIMETRIC DETERMINATION OF STARCH IN CEREAL PRODUCTS

I. RAPID DETERMINATION OF STARCH IN CRUDE GLUTEN¹

By K. A. CLENDENNING²

Abstract

Neither the original Mannich-Lenz procedure nor its recently proposed revisions proved suitable for the polarimetric determination of starch in gluten. The principal defect lay in the means of removing or correcting for proteins dissolved along with the starch and there also was a need for improvements in technique of starch dispersion.

In the present method, the starch of the gluten sample is dispersed by 15 min. boiling in a calcium chloride solution having a specific gravity of 1.30 and a pH of approximately 2.5. Frothing is controlled by the addition of one or two drops of *n*-octyl alcohol, constant volume being maintained during boiling by the addition of water. The proteins dissolved by this treatment are precipitated with 20% stannic chloride prior to filtration and polarization. This rapid procedure underestimates the starch content but the application of a factor to correct for the constant ratio of underestimation allows the real starch content to be determined with an accuracy of $\pm 0.5\%$.

Introduction

In connection with investigations on the separation of wheat flour into starch and gluten, a rapid method of determining the starch content of crude gluteins was required as a means of assessing the value of different washing procedures. From the standpoint of simplicity and rapidity, polarimetric methods appeared preferable to the diastatic and gravimetric methods, and of the numerous polarimetric techniques that have been described, that of Mannich and Lenz (7) offered numerous advantages, particularly as revised by Hopkins (2). The Mannich-Lenz procedure and its recently proposed revisions accordingly have been applied to the rapid determination of starch in crude gluten. On introducing certain modifications to overcome difficulties with frothing and proteins dissolved along with the starch, a satisfactory method has been developed from the original procedure.

Experimental

The revision proposed by Jirak (5) proved out of the question since the magnesium chloride solution prescribed as starch solvent caused the gluten to become extremely viscous. The original procedure (7) also could not be applied to the determination of starch in gluten because of the large amounts of protein dissolved along with the starch and the lack of an accurate means of correcting for them. Hopkins (2) overcame the soluble protein difficulty

¹ Manuscript received May 21, 1942.

Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as paper No. 195 of the Associate Committee on Grain Research and N.R.C. No. 1065.

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in the analysis of wheat flour by thorough extraction of the flour with 65% ethyl alcohol prior to extraction with boiling calcium chloride solution. This revision proved inadequate for the determination of starch in gluten, low inconsistent results always being obtained because of the large amounts of protein that were dissolved despite careful preliminary alcoholic extraction. Protein precipitants were therefore tested experimentally by the writer, phosphotungstic acid being chosen because of its wide use in polarimetric methods of starch determination (1, 6, 9, 10, 11) and stannic chloride because Mannich and Lenz (7) claimed that in contrast to other protein precipitants, this reagent would precipitate proteins satisfactorily without precipitating starch.

Before dealing with the removal of proteins from calcium chloride solutions containing starch, attention should be directed to difficulties that have been experienced by a number of analysts in applying the revised Mannich-Lenz procedure to the determination of starch in cereal products (3, 4, 8). Various suggestions have been made as to the heat treatment and acidity that should be employed to obtain satisfactory starch dispersal. From experimental studies to be reported in detail in a second paper, the writer has reached a number of conclusions concerning the proper conditions for the extraction and dispersal of wheat starch by boiling calcium chloride solution. For the present it will suffice to note the following relevant points.

1. The stock calcium chloride solution should have a specific gravity of 1.30 and a definitely established acidity. Prior to use, its pH must be adjusted to between 2.0 and 3.0, preferably 2.5.

2. Boiling is best conducted in 300 to 400 cc. Berzelius beakers. It should be made to proceed steadily for at least 15 min., constant volume being maintained by the addition of water.

3. To control frothing, one or two drops of *n*-octyl alcohol should be added to the calcium chloride solution.

The Precipitation of Soluble Proteins

A gluten sample known to contain 10.3% starch was ground to pass a 140 mesh sieve and a series of 2-gm. subsamples then was extracted by 15 min. boiling with 60 cc. stock calcium chloride solution + 2 cc. 0.8% acetic acid. To the cooled extracts, volumes of freshly prepared 10% phosphotungstic acid and 20% stannic chloride ranging from 0 to 15 cc. were added, after which the extracts were made to volume; mixed, filtered, and polarized in 1 dm. tubes. The protein control of the filtered extracts was determined by Kjeldahl nitrogen estimations on 25 cc. aliquots, employing a factor of 5.7 to convert nitrogen to protein content. The measurements of optical rotatory power and protein content are given in Figs. 1 and 2.

From the data presented here, it is clear that stannic chloride will remove dissolved proteins quite as efficiently as the more widely used phosphotungstic acid. The stannic chloride reagent has a decided advantage in that it does not precipitate starch when present in excess. Phosphotungstic acid will, in

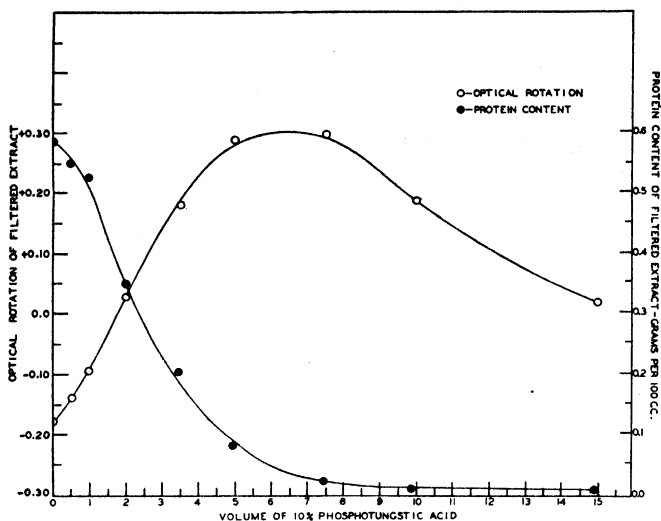


FIG. 1. The effect of increasing volumes of 10% phosphotungstic acid upon the optical rotatory power and protein content of calcium chloride extracts of gluten.

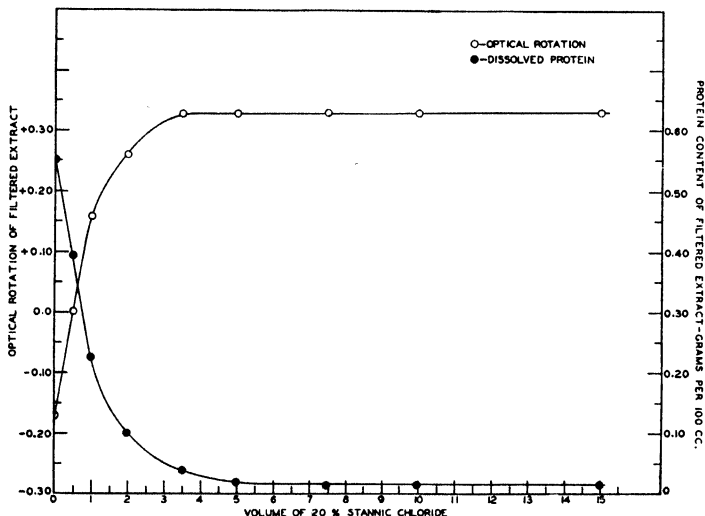


FIG. 2. The effect of increasing volumes of 20% stannic chloride upon the optical rotatory power and protein content of calcium chloride extracts of gluten.

fact, precipitate all of the starch dispersed in calcium chloride solution if added in sufficient quantity. The only undesirable effect of 20% stannic chloride consists of a tendency for gluten extracts to become cloudy on standing, when this reagent is present in large excess. As stannic chloride is highly acid in aqueous solution, starch also is apt to be degraded on standing

over long periods in its presence. Between 3.5 to 5.0 cc. 20% stannic chloride should be added to calcium chloride extracts of 2-gm. gluten samples for the satisfactory removal of dissolved proteins, judging from the data of Fig. 2. Using 3.5 cc. 20% stannic chloride upon calcium chloride extracts of 2-gm. samples of an absolutely starch free gluten, crystal clear filtrates having an optical rotation of exactly zero were obtained. This volume of stannic chloride accordingly has been adopted as a standard in the routine analysis of gluten.

The Effect of Gluten Particle Size upon Starch Extraction

The extraction of starch from gluten particles is rendered difficult by the enmeshing of the starch grains in the proteinaceous matter. Careful grinding of the material prior to extraction should facilitate the complete removal of the starch because of the resulting increase in surface. To test the effect of particle size, samples ground in the Wiley mill and ball mill were subjected to analysis by the rapid polarimetric method described below (Table I).

TABLE I

APPARENT STARCH CONTENT OF SAMPLES GROUND IN THE WILEY MILL AND BY BALL-MILLING

Sample	Wiley-milled		Ball-milled	
	α	Apparent starch, %	α	Apparent starch, %
1	0.29°	7.25	0.37°	9.25
2	0.50°	12.50	0.58°	14.50
3	0.32°	8.00	0.35°	8.75
4	0.15°	3.75	0.21°	5.25

Samples that had been ground in the Wiley mill also were separated into different particle sizes by passing through a series of sieves, the separated fractions then being similarly analysed (Table II).

TABLE II

EFFECT OF PARTICLE SIZE UPON THE EXTRACTION OF STARCH FROM GLUTEN WITH CALCIUM CHLORIDE SOLUTION

Particle size	Gluten No. I		Gluten No. II		Gluten No. III	
	α	Apparent starch, %	α	Apparent starch, %	α	Apparent starch, %
Passing:						
140 mesh	0.59°	14.75	0.45°	11.25	0.33°	8.25
85-100 mesh	0.55°	13.75	0.40°	10.00	0.29°	7.25
36-60 mesh	0.40°	10.00	0.30°	7.50	0.19°	4.75
20-36 mesh	0.35°	8.75	0.26°	6.50	0.15°	3.75

The effect of particle size upon the amount of starch extracted by 15 min. boiling apparently is a very marked one. Subsequent tests showed that this effect as it appears in Table II is not caused by differences in the starch content of various sized particles. From this it is clear that grinding the samples to pass a 140 mesh sieve should be adopted as a standard practice in the preparation of all samples for analysis.

Effect of Extraction Time upon Starch Recovery

The foregoing data, as well as showing the effect of particle size upon the amount of starch extracted in a 15 min. extraction period, indicated that the starch content of gluten ground to pass 140 mesh might be underestimated through incomplete extraction. To test the effect of extraction time upon starch recovery, duplicate 2-gm. samples of a series of 140 mesh gluten samples of known starch content were extracted for 15, 40, and 60 min., removing the dissolved proteins as usual with stannic chloride. The polarimetric readings are recorded below in Table III.

TABLE III

EFFECT OF EXTRACTION TIME UPON THE COMPLETENESS OF STARCH RECOVERY FROM GLUTENS OF KNOWN STARCH CONTENT

Sample No.	Optical rotation for 100% recovery	Optical rotation with 15 min. extraction	Optical rotation with 40 min. extraction	Optical rotation with 60 min. extraction
1	+ 0.18°	0.15°	0.15°	0.17°
2	+ 0.36°	0.30°	0.33°	0.33°
3	+ 0.55°	0.45°	0.51°	0.52°
4	+ 0.73°	0.60°	0.71°	0.71°

In assessing the significance of the data of Table III the effect of increased duration of boiling upon several different factors contributing to the extract's optical activity must be borne in mind. The precipitation of the proteins dissolved by 15 min. extraction is not absolute, sufficient remaining (0.04 gm. per 100 cc.) to cause a negative reading of 0.003°, and with a longer period of boiling, this error should tend to increase. Increased duration of boiling also should reduce the specific rotation of wheat starch judging from the data of Mannich and Lenz (7). These two effects, however, should not cause a negative error totalling more than -0.01°. There remains the possible occlusion of starch on the stannic chloride-protein precipitate. In view of the large amount of protein precipitated, a small attending loss of dissolved starch is regarded as inevitable.

Because of the possible effects of residual dissolved proteins, altered specific rotation with prolonged boiling, and starch losses attending the removal of

proteins, it is not surprising that the optical rotatory power of extracts obtained by 60 min. boiling is below the theoretical values given to represent 100% recovery. The values in fact are low by 0.01 to 0.03° in the 60 min. extraction series. This small bias is attributed largely to starch occlusion on precipitated proteins. With 15 min. extraction, the optical rotation values are low by 0.03 – 0.13° , depending on the starch content of the sample. This increased discrepancy is attributed to an incomplete extraction of the starch.

Since the operator must devote almost continuous attention to samples during their extraction in stirring, maintaining constant boiling rate and volume, and rubbing down the sides of the beakers, an extraction period of more than 15 min. increases the labour required per determination very considerably. Lengthening the procedure in order to reduce the correction factor is in the author's opinion unwarranted for the purposes of routine analysis. In the rapid procedure outlined below, in which 15 min. extraction is used in conjunction with a correction factor, an experienced technician should be able to determine the starch content of 12 or more gluten samples in six hours with little difficulty.

Calculation of the Ratio of Underestimation of Starch Content

For this purpose, several hundred grams of gluten was completely freed of starch grains, as judged by microscopic observation, through careful and prolonged machine and hand washing. After air-drying and grinding to pass 140 mesh, calcium chloride extracts of this material failed to give a starch test with iodine. The dry finely ground gluten was then divided into five subsamples and to these wheat starch of known purity was added to give mixtures containing 0 to 18% starch. After careful mixing in the dry state, distilled water was added slowly with continuous stirring until a lump of moist dough was obtained containing all of the gluten and starch of the subsample. After careful kneading for half an hour in the dough state, the mixtures were drum-dried and ground again to pass a 140 mesh sieve. The weight of starch added to each subsample, less known impurities, was used in calculating the *actual* starch content of each sample. The *apparent* starch content was determined polarimetrically, employing the equation of Mannich and Lenz to convert the observed reading of optical rotatory power to starch content (Fig. 3).

The relationship of the apparent to the actual starch content is seen to be linear over the range 0 to 18% starch content and is represented by the equation: % present = 1.21 (% found). Because of the constancy of the ratio between the actual and apparent starch content, the rapid determination of the latter should give an accurate measure of the true starch content when multiplied by the factor 1.21. Employing this correction factor, analyses were conducted on two glutes prepared in another laboratory by the method given above so as to contain 8 and 16% starch, respectively. Agreement between the actual and determined starch content then was within $\pm 0.3\%$. The starch content of a crude machine-washed gluten was determined by a diastatic technique which took full cognizance of the effect of the gluten upon

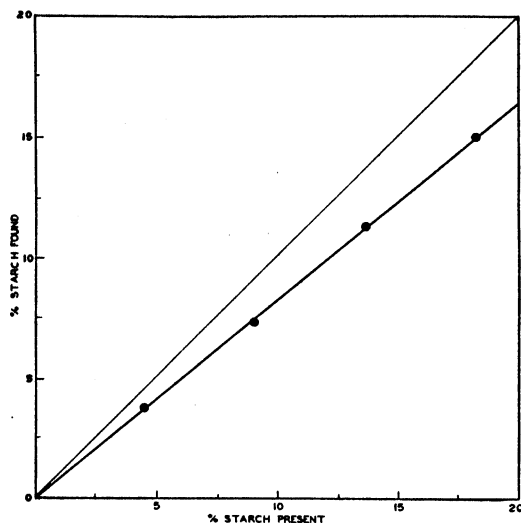


FIG. 3. Relation of actual starch content of crude gluteins to starch content determined polarimetrically.

the final sugar determinations. Agreement within 0.3% was again observed when this gluten was analysed by the rapid technique described below.

Method

Reagents

(1) Concentrated calcium chloride solution

Dissolve two parts of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ in one part of water. Adjust the specific gravity to 1.30. Adjust the pH to 5.50 with dilute hydrochloric acid and sodium hydroxide. In preparing this stock solution only reagent quality chemicals should be used. Filter with suction until crystal clear before using.

(2) 20% Stannic chloride solution

Dissolve 20 gm. $\text{SnCl}_4 \cdot 5\text{H}_2\text{O}$ by warming with about 80 cc. of the above calcium chloride solution. Cool and make to volume with stock calcium chloride solution in a 100 cc. volumetric flask. After mixing and filtering, this reagent should be crystal clear. Unlike many protein precipitants, this reagent does not have to be freshly prepared before use.

(3) 0.8% Acetic acid

(4) *n*-Octyl alcohol

(5) 95% Ethyl alcohol

Procedure

Weigh 2.0 gm. of the dry 140 mesh gluten into a 300 to 400 cc. Berzelius beaker. Add 60 cc. calcium chloride solution and 2 cc. 0.8% acetic acid, omitting the initial treatment with water required in the analysis of cereal

products of high starch content. Immediately whip the contents of the beaker gently until a uniform suspension is obtained. All lumps must be completely disintegrated at this stage. Add two drops *n*-octyl alcohol for frothing control. After marking the level of the beaker's contents with a wax pencil, bring to boiling in approximately five minutes, stirring constantly. It is our practice to use a 3 in. non-luminous flame of the Cenco Tirril type burner, protected by an earthenware burner guard.

When boiling is proceeding briskly, reduce the flame by one-half inch and maintain gentle but steady boiling for 15 min. During this period of boiling, the mixture should be stirred occasionally, taking care at all times to keep the gluten particles rubbed down into the solution from the sides of the beaker by the use of a rubber tipped stirring rod. Water should be added with stirring from time to time so as to maintain a constant boiling volume. After 15 min. boiling, cool to room temperature in running tap water. Pour the contents of the beaker into a 100 cc. volumetric flask containing 3.5 cc. 20% stannic chloride solution. After shaking the contents of the volumetric flask, complete the transfer by successively rinsing the beaker with stock calcium chloride solution. Destroy any foam by adding one or two drops of 95% ethyl alcohol. Now make to volume accurately with the calcium chloride solution. Shake vigorously for three minutes. Allow 10 cc. to run through a fluted 15 cm. Whatman No. 12 filter paper, taking care to wet the whole filtering surface thoroughly. Discard this first filtrate, and then filter the remainder of the solution without suction. Polarize the crystal clear filtrate in a 1 dm. tube. In the present work, polarization has been by means of an Adam Hilger triple-field polarimeter, using either a Zeiss sodium vapour lamp or an Adam Hilger F289 sodium burner as a source of sodium light. The starch content of the crude gluten can then be calculated from the formula
$$\frac{(50 \alpha) (1.21)}{(\text{wt. of sample in gm.})} = \% \text{ starch, where } \alpha = \text{observed optical rotation in degrees.}$$

Acknowledgments

The author is indebted to Dr. W. H. Cook, Dr. G. A. Adams, and Dr. J. W. Hopkins for numerous helpful suggestions. It is also a pleasure to acknowledge the assistance of Mr. D'Arcy Wright throughout the present work.

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INTERFERTILITY STUDIES AND INHERITANCE OF LUMINOSITY IN *PANUS STYPTICUS*¹

BY RUTH MACRAE²

Abstract

Examination of five collections of *Panus stypticus* (Bull.) Fries from Europe and of 10 collections from North America has corroborated the findings of other workers that the European form of this fungus is non-luminous and the American form luminous. Series of pairings in all possible combinations of monosporous mycelia from single fruit bodies have shown that both forms are heterothallic and tetrapolar. Pairings between monosporous mycelia of different collections of the American and European forms are fertile. A study of diploid mycelia and fruit bodies of the F_1 generation from crosses between the luminous and non-luminous forms, and of haploid mycelia from an F_1 fruit body produced by such a cross, has shown that luminosity in this species is an inherited character, that it is governed by a single pair of Mendelian factors in which luminosity is dominant over non-luminosity, and that the luminosity factors form all possible combinations with the interfertility factors. A point of special interest in the study of the inheritance of this factor is in its expression as a dominant character in the dikaryotic mycelium and fruit bodies of the F_1 generation.

Since the discovery of heterothallism in the Basidiomycetes, genetical studies in these fungi have dealt almost exclusively with the sexuality or interfertility factors and related problems. Although in more recent years much work has been done on the inheritance of morphological and pathological characters and the origin of physiologic races through mutation and hybridization in the Uredinales³ and Ustilaginales⁴, in the Hymenomycetes the inheritance of factors other than the interfertility factors has been studied in only a few species. The work that has been done in the latter group along this line is reviewed briefly here.

Vandendries and Brodie (52) and Brodie (5) have reported on what they have called the "barrage" phenomenon in *Lenzites betulina* (L.) Fr., a tetrapolar species. They found that in this and other species when certain haploid⁵

¹ Manuscript received May 11, 1942.

Contribution No. 705 from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada. Part of a thesis presented to the School of Graduate Studies, University of Toronto, Toronto, Ont., in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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³ Buller (7, 8); Craigie (10); Gordon and Welsh (18); Gassner and Straib (16); Johnson and Newton (22, 23, 24, 25); Johnson, Newton, and Brown (26, 27); Levine and Colter (32); Newton and Johnson (37, 38, 39); Newton, Johnson, and Brown (40, 41); Stakman, Levine, and Colter (49, 50); Waterhouse (53, 54).

⁴ A review of the genetics of the Ustilaginales with a list of references is given by Christensen and Rodenhiser (9).

⁵ In the heterothallic Basidiomycetes the mycelium arising from a single spore is referred to as a monosporous, haploid, or monokaryotic mycelium or a haplont since it possesses nuclei with the single or haploid number of chromosomes arranged singly, that is as monokaryons, within the cells. The mycelium produced by the union of two compatible haploid mycelia is called a diploid or dikaryotic mycelium or a diplont because the haploid nuclei which it contains are associated together in pairs or dikaryons within the cells and each cell, therefore, contains the diploid or double number of chromosomes. An explanation of the use of these and other associated terms has recently been given by Buller (7, 8).

mycelia were grown side by side on nutrient agar, the two mycelia exhibited a mutual aversion resulting in the formation of a zone or barrage between them which was bridged by only a few hyphae or none at all. The authors found that the presence of the barrage in *L. betulina* was correlated with the genetic constitution of the mycelia concerned and appeared when one pair of the interfertility factors, designated by the authors as the *Bb* factors, was unlike. Its manifestation was, on the other hand, completely independent of the other pair of interfertility factors.

Biggs (4), examining the pairing reactions of 25 monosporous mycelia of a single fruit body of *Cytidia salicina* (Fr.) Burt, observed that both the barrage phenomenon and the formation of false clamp connections in this species occurred when the paired mycelia possessed in common one of the interfertility factors, arbitrarily designated by her as the *B* or *b* factor. In this species the factors governing the formation of a barrage and those controlling the production of false clamp connections are evidently both associated with the same pair of interfertility factors.

Quintanilha (44) in *Coprinus fimentarius* Fr. and Routien (48) in *Coprinus plicatilis* Fr. found that false clamp connections were formed in these species when the *A* or *a* factors of the paired monosporous mycelia were different and the *B* and *b* factors alike.

Kaufert (28) noted four types of reactions between haploid mycelia from single sporophores of the tetrapolar species *Pleurotus corticatus* Fr. These types of reactions were correlated with the similarity or dissimilarity of the interfertility factors in the paired haplonts. In the compatible pairings where the mycelia contained both sets of interfertility factors ($AB \times ab$, $Ab \times aB$) and in the incompatible pairings where the mycelia had the same pair of interfertility factors ($AB \times AB$, $ab \times ab$, $Ab \times Ab$, $aB \times aB$) there was a complete intermingling of the mycelia of the two haplonts at the line of junction between them. In the incompatible pairings where the *A* factors were the same and the *B* factors different ($AB \times Ab$, $ab \times aB$) a sharp line of demarcation, consisting of a narrow white line of aerial mycelium, was produced where the hyphae of the two mycelia mingled but did not grow further into the area occupied by the other haplont. Where the *B* factors were the same and the *A* factors different ($AB \times aB$, $ab \times Ab$), the hyphae of the two mycelia intermingled freely and anastomoses occurred but there was an almost complete inhibition of coremial and conidial formation in both mycelia.

In all these investigations the characters described were found to be closely associated with the interfertility factors. In the investigations reviewed below, other characters have been shown to form all possible combinations with the interfertility factors and the dominance of the character or its allelomorph to be manifested when the two nuclei became associated as a dikaryon in the diploid mycelium.

Zattler (55) paired with each other haploid mycelia of two varieties of *Collybia velutipes* (Curt.) Fr., one with dark brown and the other with white mycelium. He found that the diploid mycelium formed by the paired

haplonts was brown in colour and that the haplonts isolated from a fruit body produced by the diploid mycelium were of four kinds: dark brown, light brown of two different intensities, and white. From his observations he concluded that the colour of the mycelium was governed by two pairs of Mendelian factors Rr and Vv which formed all possible combinations with the interfertility factors. The presence of either of the two dominant factors, R or V , gave to the mycelium a brown coloration but R gave a more intense colour than V , hence Rv haplonts were intense brown, Rv and rV haplonts light brown of different intensities, and rv haplonts white.

Working with a Canadian strain of *Schizophyllum commune* Fr., the same author found that when certain haploid mycelia were paired the diploid mycelium produced abnormal ball-shaped fruit bodies, "Knäuel-Fruchtkorper", but when other haploid mycelia of the same strain were paired the diploid mycelium gave rise only to normal fruit bodies. He judged from his experiments that in this Canadian strain normal fruit body formation was governed by a single pair of Mendelian factors Gg , the factor G for normal fruit body formation being dominant over the factor g for abnormal fruit body formation.

Gilmore (17) found that in *Psilocybe coprophila* Fr. the production of abnormal fruit bodies of various types was more preponderant in two of the crosses than it was in other crosses of monosporous mycelia from a single fruit body. She came to the conclusion that this character was inherited but could not be expressed in a simple Mendelian ratio.

Dickson (11, 12, 13) made a study of the morphological characters of haploid and diploid mycelia of *Coprinus sphaerosporus*, their growth rates and the production of fruit bodies in culture. He found that the F_1 generation from any cross consisted of several types with respect to these characters and from his experiments came to the conclusion that one of the types "is of the original 'wild type' and contains the full chromosome complement combined with a large percentage of dominant genes". The other types found, he suggests, may have contained a number of recessive factors and/or certain chromosome abnormalities such as translocations, deletion, or inversion which resulted in morphological and physiological types different from both parents.

Quintanilha and Balle (45, 46, 47) found that some of the spores of certain fruit bodies of *Coprinus fimentarius* gave rise to vigorous mycelia with abundant branching and fairly rapid rate of growth, and others to poorly developed mycelia varying from a single short hypha or a small swelling to a vesicular, branched mycelium of very slow growth. They called these two types "normal" and "dwarf" mycelia. Among the fruit bodies producing normal and dwarf mycelia, they found one in which every tetrad examined (101 in all) produced two normal and two dwarf mycelia. When two normal haploid mycelia from this fruit body were paired, only normal mycelia were produced in the next generation; a dwarf mycelium paired with a normal mycelium gave rise to two normal and two dwarf mycelia in each tetrad of the next generation; pairings of two dwarf mycelia never succeeded. They explain these results by the presence of a pair of Mendelian factors Nn ,

where N is responsible for normal growth and n is its sublethal allelomorph. They have remarked that in the dikaryophase N is completely dominant over n and the heterozygous diploid mycelia Nn and the fruit bodies that they produce are indistinguishable from the homozygous dominants NN . They were not able to show an association of the factors for dwarfness with the interfertility factors but found that these factors were distributed equally among the four interfertility groups and concluded that the three pairs of factors must be situated on three different pairs of chromosomes.

From their observations on dwarfness in *C. fimentarius* Quintanilha and Balle are convinced of the existence of a series of multiple allelomorphs (N, n, n_1 , etc.) of sublethal genes, each one responsible for a special type of dwarfness. In two fruit bodies they found evidence of irregular disjunction: in addition to the numerous tetrads of two normals plus two dwarfs, many others gave three dwarfs and one normal, one dwarf and three normals, four more or less dwarf, or four nearly normal. Genetical analysis of this type of dwarfness was not finished when they published their results but they concluded that it was a case of hereditary dwarfness probably conditioned by a single pair of Mendelian factors but varying greatly in its manifestation due to environmental conditions or the arrangement of the chromosomes within the cell itself.

While working with *Peniophora Allescheri* Bres., Nobles (42) obtained a mutant haploid mycelium which was entirely unlike the normal haploid mycelium in rate and type of growth and production of conidia. The normal mycelium was slow growing with scanty aerial hyphae and numerous conidiophores; the mutant mycelium had a rapid rate of growth, abundant aerial hyphae, and lacked conidiophores. When the mutant mycelium was paired with a normal haploid mycelium the diploid mycelium produced was like the mutant parent in its rapid rate of growth and abundant aerial hyphae but like the normal parent in that conidiophores and conidia were produced. It was found that the conidia of this species had only one nucleus and so in order to ascertain if the nuclei of both parent types were present in the hybrid mycelium, 295 mycelial colonies from the conidia of the hybrid mycelium were examined; 153 of these were found to be identical with the mutant parent and 142 with the normal parent. In this species, "the two nuclei of a pair, though existing independently in the cells, coöperate in determining the characters of the diploid colony".

In the investigations described in the present paper an additional example is given of the inheritance of a dominant character in the Hymenomycetes. The fungus studied is *Panus stypticus* (Bull.) Fries, a small coriaceous agaric which grows in groups or clusters usually on the wood of deciduous trees and very rarely on coniferous hosts. Collections of this fungus from North America differ from those of European origin in that the former are luminous and the latter non-luminous. Polysporous cultures and series of monosporous cultures were obtained from the fruit bodies of collections from North America and Europe, the fruit bodies and cultures examined for luminosity, and the

different series of monosporous isolates paired by themselves and with each other in order to determine the relationship between the luminous and non-luminous forms of the fungus and the inheritance of the factor for luminosity in a cross between them.

Materials and Methods

A list of the collections from which cultures were made, together with the hosts and localities from which they came, is given in Table I.

TABLE I

Collection number	Host	Locality
American		
1532	<i>Quercus rubra</i>	Timagami, Ont.
2189	<i>Acer</i> sp.	Ann Arbor, Mich.
2190		Ste. Anne de Bellevue, Que.
2191		New Haven, Conn.
5105	<i>Alnus incana</i>	Ottawa, Ont.
6004	<i>Betula papyrifera</i>	Lake MacDonald, Que.
7543	<i>Acer</i> sp.	Chelsea, Que.
7544	<i>Alnus incana</i>	Eardley, Que.
8303	Deciduous stump	Otter Lake, near Huberdeau, Que.
9518	<i>Alnus incana</i>	Ottawa, Ont.
European		
1667	<i>Fagus</i> sp.	Beiersdorf, near Dresden, Germany
2118	<i>Quercus pedunculata</i>	Darmstadt, Germany
2188	<i>Salix</i> sp.	Sigless, Burgenlande, Austria
5120	<i>Quercus</i> sp.	Potsdam, Germany
5122		Wageningen, Holland

Polysporous and monosporous cultures were obtained from single fruit bodies of each of these collections in the following manner. A fruit body was moistened and suspended over a sterile glass slide. From the spore deposit thus obtained, spore dilutions in sterile distilled water were made and spread over the surface of a gelatine medium, containing 10% gelatine, 1% lactose, and 1% dextrose, in Petri dishes. When the spores had germinated, monosporous cultures were isolated by cutting out, with a fine needle under the compound microscope, a square of gelatine containing a single sporeling and placing it in a tube of malt agar. Polysporous cultures were obtained by transferring a square of gelatine containing a number of germinating spores to a tube of malt agar.

Most pairings were made by placing small pieces of two monosporous or haploid mycelia about one centimetre apart on slants of malt agar in test-tubes; some, however, were made in Petri dishes containing malt agar. Two weeks or more after inoculation the mycelium at the junction of the two haploid mycelia was removed, mounted in a 7% aqueous solution of potassium hydroxide, stained with an aqueous solution of phloxine, and examined for clamp connections.

In the accompanying tables of pairings, with the exception of the table in Fig. 9, the sign in each square represents the result of a single pairing between two haploid mycelia. The plus sign indicates that the two haploid mycelia had united to form a diploid mycelium with clamp connections, i.e., the mycelia were interfertile or compatible. The minus sign indicates that no clamp connections were found in the pairing, i.e., the two mycelia were intersterile or incompatible. The table in Fig. 9 differs from the other tables in that the sign in each square represents the results of a series of pairings between a number of haploid mycelia from two different collections, rather than of a single pairing between two haploid mycelia.

Whether the cultures and fruit bodies were luminous or non-luminous was determined by examining them after 15 to 30 min. in a dark-room when the eyes had become accustomed to the darkness and able to perceive a dim light.

Luminosity

Probably the first record of luminosity in *Panus stypticus* was made by Ellis (15) who writes: "Some time last fall (1885) Prof. Thos. G. Gentry, of Philadelphia, Pa., called my attention to the fact that *Panus stypticus*, Fr., is phosphorescent. Prof. G. had collected some specimens of this species and laid them with other fungi on a shelf to dry. On examining the specimens the same evening, it was found that the gills of the *Panus* were distinctly phosphorescent, a fact which I have been able to verify by my own observation of specimens, soon after collected at Newfield. By careful examination, the luminosity was found to proceed from the gills and not from the stipe, nor from the upper surface of the pileus, nor, finally, as was at first suspected, from any fragment of rotten wood attached to the specimen. This phosphorescence was not observed in all specimens brought in for examination, and seemed to depend on some peculiar condition of the air, having been noticed only in specimens gathered in damp weather or just before a storm."

Atkinson (3), Hard (20), Murrill (36), and Kauffman (29) describing collections of *P. stypticus* found in North America, state that the fungus is "phosphorescent". Molisch (35), commenting on the findings of Gentry and Ellis, says that he had collected *P. stypticus* repeatedly around Vienna, Austria, and had never seen luminescence in the fruit bodies, nor were pure cultures of the mycelium on bread luminous. Miss Johnson (21) working in England on the biology of *P. stypticus* reported that no fruit bodies that she had examined were luminous.

Buller began his observations on the luminosity of *P. stypticus* in 1910 and published in 1924 a full account of his investigations. He reports that all the collections from North America that he had examined were luminous, but that those from England were non-luminous. From his investigations he concludes that there are two distinct physiological forms of the fungus: one in North America having luminous fruit bodies which he calls *Panus stypticus luminescens*, and another in England having non-luminous fruit

bodies, to which he gives the name *Panus stypticus non-luminescens*. He says (6) that "Since luminous fruit-bodies of *Panus stypticus* have never been recorded by any European mycologist, it seems extremely probable that *P. stypt. non-luminescens* is not confined to England but occurs throughout Europe. Probably its distribution is Eurasian in extent. It seems most likely that one of the two physiological forms arose from the other and that to-day they are separated geographically because the great barrier of the Atlantic Ocean prevents their intermingling."

The writer has examined five collections of *P. stypticus* from Europe, three from Germany, one from Austria, and one from Holland (Table I). None of the fruit bodies of the five collections were luminous, nor were the polysporous or monosporous mycelia originating from them. These observations, in addition to Molisch's findings for collections from Vienna, agree with Buller's supposition that *P. stypticus* found on the continent of Europe, like the form found in England, is non-luminous.

Studying the luminosity of the American form of *P. stypticus* Buller found that the fruit bodies when immature were luminous all over their surfaces but when mature the luminosity was confined to the gills. He observes that "A fruit-body is most strongly luminous when it is just full-grown. Thereafter, as it exhausts itself by shedding spores its light gradually declines, finally becoming invisible shortly before the end of the spore discharge period." The fruit bodies of *P. stypticus* absorb moisture readily and may be revived after drying. Those that have not passed maturity and are still luminous lose this luminosity when dried but regain it when moistened. This occurs frequently throughout the growing season of the fungus and may explain the observation made by Ellis that "phosphorescence was not observed in all specimens brought in for examination and seemed to depend on some peculiar condition of the air, having been noticed only in specimens gathered in damp weather or just before a storm."

Buller noted that when wood collected with the fruit bodies was moistened and left for several hours or days the mycelium then growing at the surface was luminous, although "sometimes pieces of wood bearing fruit-bodies never showed the least sign of luminescence." (Compare Gentry's observation quoted above.) The writer has observed that if the wood on which the fungus is actively growing and producing fruit bodies is sufficiently moist, the mycelium in it is brightly luminous. It would seem that when fruit bodies and mycelium are physiologically active they are luminous, but if, through dryness or for any other reason, activity ceases, luminosity is curtailed.

In all 10 collections of *P. stypticus* from North America examined by the writer luminous fruit bodies were found. In six of the collections all the fruit bodies were luminous. In a seventh collection, No. 1532, the one fruit body examined was found to be luminous. Most of the fruit bodies of another collection, No. 2191, were luminous, a few were not. No attempt was made to obtain spores from the non-luminous fruit bodies of this collection but it is

possible that they were no longer luminous because their activity had ceased. In the two remaining collections, Nos. 7544 and 8303, both luminous and non-luminous fruit bodies were present, all actively shedding spores. It is not known if the non-luminous fruit bodies of these last two collections were nearing the end of their spore discharge period and so had lost their luminosity, although the abundance of spores given off would seem to preclude this possibility, or if the physiological vigour of the fruit bodies had been affected by adverse conditions and luminosity curtailed. That these fruit bodies were from a non-luminous strain seems unlikely since some of the fruit bodies of the same collection were brightly luminous and the partial recovery of luminosity by two of the non-luminous fruit bodies of collection No. 8303 suggests that the non-luminous fruit bodies of this collection may have been luminous originally. Moreover, at least some of the monosporous cultures and all the polysporous cultures derived from each of the non-luminous fruit bodies of the two collections were luminous. In the non-luminous strain from Europe, fruit bodies, monosporous cultures, and polysporous cultures are all consistently non-luminous.

Studying the luminosity of monosporous and polysporous mycelia of the American form of *P. stypticus* in culture, the writer has found that they gradually lose their luminosity with age and consequent loss of activity but regain it when transferred to fresh media. The monosporous cultures isolated from a single fruit body were found, even when first isolated, to vary in the intensity of light produced and a few monosporous cultures isolated from luminous fruit bodies were found to be non-luminous from the beginning. Many of the monosporous mycelia lost their luminosity permanently a few weeks or a number of months after isolation and only a comparatively few have kept it throughout the time of these experiments. All the polysporous cultures, however, have retained their luminosity up to the present time. The polysporous cultures of any fungus are generally more vigorous than the monosporous cultures and it would seem here that the more vigorous cultures have retained their luminosity.

To determine whether or not the spores were luminous, heavy spore deposits from three luminous fruit bodies on glass slides and another spore deposit from a brightly luminous fruit body on malt agar were examined in the dark but no trace of luminosity was visible. The spore deposit on malt agar was examined on the first and second days after germination of the spores had taken place but again no luminosity could be seen. Ten days after germination, however, the young mycelial colony was brightly luminous. The non-luminosity of the spores and young mycelia was noted by Buller who suggests that the very young mycelia may be luminous but so faintly so as to be invisible to the human eye.

A monosporous and a polysporous culture of collection No. 6004 of the luminous American form of *P. stypticus* and a diploid culture, XLIV, produced by pairing a luminous monosporous culture, No. 6004-6, with a non-luminous monosporous culture, No. 5122-20, from a European collection were given

PLATE I

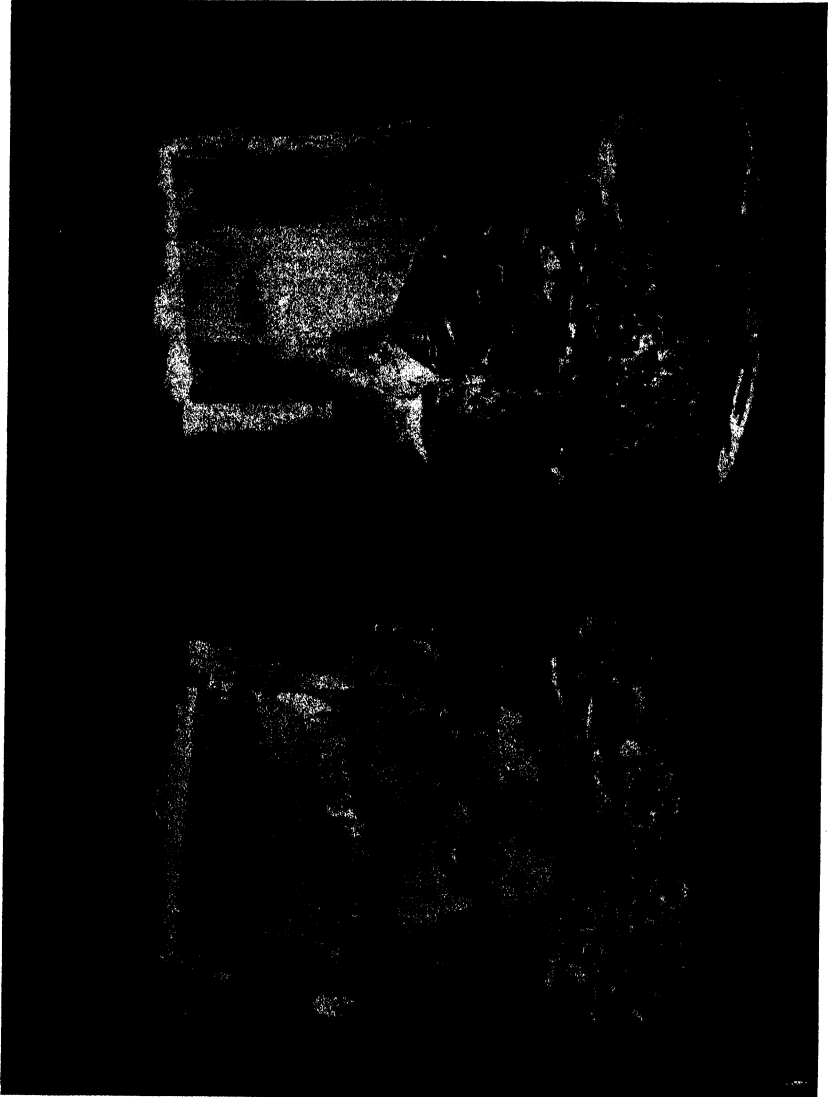
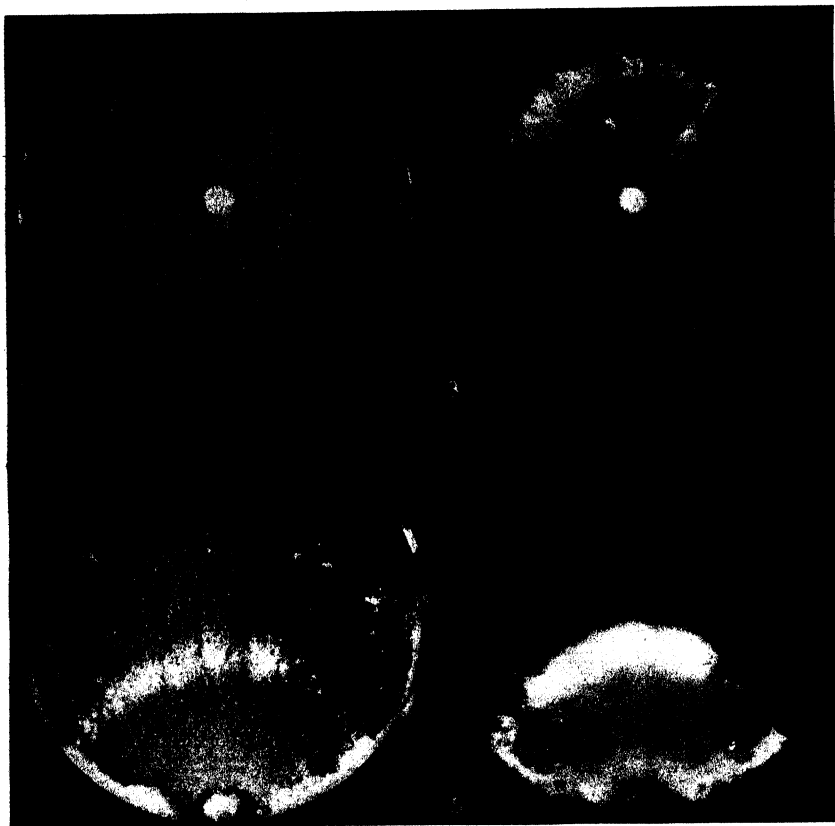


FIG. 1. *Penus stypticus*. Hybrid fruit bodies produced by the diploid mycelium from a pairing between two monosporous mycelia of the luminous American and non-luminous European forms, 0004-6 \times 5122-20, grown on a section of white birch resting on sphagnum moss in a wide-mouthed jar. Photographs show both sides of the same culture. $\times 4$.

PLATE II



Panus stypticus cultures grown on 1.25% malt agar. $\times \frac{3}{4}$ approximately. FIG. 2. A monosporous culture, No. 6004-6, five weeks old, photographed by reflected light. FIG. 3. The same culture as Fig. 2 photographed by its own light. FIG. 4. A polysporous culture, No. 6004, six weeks old, photographed by reflected light. FIG. 5. The same culture as Fig. 4 photographed by its own light.

to Dr. L. E. Howlett of the Division of Physics and Engineering of the National Research Council of Canada. He very kindly made photographs of the spectra of the luminescence of all three cultures with a helium spectrogram for comparison, and reported concerning them: "Light was emitted from all three in the region 4900–5800 Å. Samples marked 6004–6 and 6004 exhibited much stronger fluorescence than the other one, XLIV, and showed a strong emission peak at about 5400–5500 Å. This peak was not nearly as apparent in sample XLIV. Samples 6004–6 and 6004 were given an exposure of 120 hours. Sample XLIV was given an exposure of 210 hours. A Zeiss high power spectrograph with an F2.3 aperture on the camera was used."

The spectrograms of these three cultures are reproduced in Plate IV. As was to be expected the three spectrograms are similar. They extend from near the beginning of the yellow region through the green and fade out in the blue green, with the peak in the yellow green region. These spectra are very like that photographed by Molisch (35) for the luminescence of the mycelium of an unidentified fungus, mycelium X. The spectrum for this fungus extends from 4800 to 5700 Å. (*P. stypticus*—4900 to 5800 Å.) The spectrum for the luminescence of *Armillaria mellea* on the other hand was found by Ludwig (33) to cover the region from 4600 to 5400 Å, i.e., from the middle of the yellow region through the green into the blue. It would appear that the wave length of the light given out by luminous fungi may vary for different species.

Photographs of the monosporous culture No. 6004–6 and the polysporous culture No. 6004, which were used for the helium comparison spectra, were taken by reflected light and in a totally dark room where the only light acting on the photographic plate was that of the luminous mycelium (Plate II). For the photographs of the cultures taken by their own light Wratten hypersensitive panchromatic plates and a lens aperture of F6.3 were used with an exposure of 26 and 50 hr. respectively.

Interfertility Studies

Series of pairings in all possible combinations of monosporous mycelia from single sporophores of collections of *P. stypticus* of North American and European origin have shown that both the luminous American and non-luminous European forms of this fungus are heterothallic and tetrapolar. These results were published by the writer in the Progress Report of the Dominion Botanist for the years 1931–1934 and in Nature (34) and have been confirmed by Vandendries (51) for the European form.

In Table II the results of pairings in all possible combinations of monosporous mycelia of three collections from North America and of five collections from Europe are summarized.

In Figs. 6 to 8 the results for the American collections Nos. 6004 and 5105 and the European collection No. 5122 are presented graphically. In these tables the four groups into which the monosporous cultures resolve themselves are designated by the factors *AB*, *ab*, *Ab*, and *aB*, which, following the usage

TABLE II

THE RESULTS OF SERIES OF PAIRINGS IN ALL POSSIBLE COMBINATIONS BETWEEN MONOSPOROUS CULTURES FROM SINGLE FRUIT BODIES OF *Panus stypticus*

Culture number	Age when paired	Number of cultures	Number of cultures				
			Group AB	Group ab	Group Ab	Group aB	Group ab + aB
American							
1532	38 days	9	3	3	1	2	
5105	62 days	22	10	8	3	0	1
6004	79 days	28	7	4	8	9	
European							
1667	52 days	9	5	1	2	1	
2118	41 days	9	4	3	1	1	
2188	65 days	10	2	4	3	1	
5120	29 days	27	4	4	12	7	
5122	1 year 213 days	13	3	3	5	2	

		AB				ab				Ab				aB															
		1	2	4	12	15	16	20	5	22	23	27	3	6	7	9	11	21	24	30	8	10	13	14	17	25	26	28	29
AB	1	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	12	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	15	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ab	16	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	20	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	22	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	23	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ab	27	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
aB	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	8	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	13	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	14	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	17	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	25	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	26	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	26	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	26	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	29	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-

FIG. 6. Table showing the results of pairing, in all possible combinations, a series of 28 monosporous cultures from a single fruit body of an American collection of *Panus stypticus*, No. 6004.

		A'B'			a'b'			A'u'				a'b'		
		1	10	19	12	14	15	21	22	23	24	26	13	20
A'B'	1		-	-	+	+	+	-	-	-	-	-	-	-
	10	-		-	+	+	+	-	-	-	-	-	-	-
	19	-	-		+	+	+	-	-	-	-	-	-	-
a'b'	12	+	+	+		-	-	-	-	-	-	-	-	-
	14	+	+	+	-		-	-	-	-	-	-	-	-
	15	+	+	+	-	-		-	-	-	-	-	-	-
A'u'	21	-	-	-	-	-	-		-	-	-	-	+	+
	22	-	-	-	-	-	-	-		-	-	-	+	+
	23	-	-	-	-	-	-	-	-		-	-	+	+
	24	-	-	-	-	-	-	-	-	-		-	+	-
a'b'	26	-	-	-	-	-	-	-	-	-	-		+	+
	13	-	-	-	-	-	-	+	+	+	+	+		-
	20	-	-	-	-	-	-	+	+	+	-	+	-	

FIG. 7. Table showing the results of pairing, in all possible combinations, a series of 13 monosporous cultures from a single fruit body of a European collection of *Panus stypticus*, No. 5122.

		AB										ab					Ab				aB		
		1	2	3	6	11	12	13	14	19	21	4	5	7	8	10	15	16	17	18	20	22	9
AB	1	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	+
	2	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+
	6	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	+
	11	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	+
	12	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	+
	13	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	+
ab	14	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	+
	19	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	+
	21	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	+
	4	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	5	+	+	-	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	7	+	+	-	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	8	+	+	-	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Ab	10	+	+	-	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	15	+	+	-	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	16	+	+	-	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	17	+	+	-	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
aB+aB	9	+	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+

FIG. 8. Table showing the results of pairing, in all possible combinations, a series of 22 monosporous cultures from a single fruit body of an American collection of *Panus stypticus*, No. 5105.

of Kniep (30), represent the pairs of allelomorphic factors that are assumed to control the pairing reactions of the monosporous mycelia in tetrapolar forms. The table for the series of pairings of collection No. 5105 is given because this series and that for collection No. 5122 are the only two of eight made that did not show complete fertility between monosporous cultures of compatible groups. This is a repetition of similar instances of incomplete incompatibility found by other workers in other species.

In Fig. 8 it may be seen that culture No. 5105-9 has, with one exception, paired with all the monosporous cultures of the two groups having the interfertility factors AB and Ab respectively. It has been assumed that two spores were picked up together when the culture was isolated and that it is composed of the haploid mycelia ab and aB which, being incompatible, have produced no clamp connections until paired with the compatible mycelia AB and Ab . The explanation, however, may be that there has been a mutation of the B factor in this mycelium and that the mycelium now has the interfertility factors aB' . No attempt was made to isolate the diploid mycelium from these pairings. There is also the possibility, although the writer has not seen such basidia in fruit bodies of *P. stypticus*, that the spore from which culture No. 5105-9 originated may have been produced on a three-spored basidium and have received two incompatible nuclei from the four nuclei present in the basidium after reduction division.

In addition to series of pairings between monosporous mycelia isolated from single sporophores, pairings were made between monosporous mycelia from different collections, i.e. between American and American collections, between European and European collections, and between American and European collections. A list of these pairings with their results is given in Table III. The results are presented graphically in Fig. 9 where the sign in each square represents the results of a series of pairings between mono-

		NORTH AMERICAN (LUMINOUS)					EUROPEAN (NON-LUMINOUS)				
		1532	2190	2191	5105	6004	1667	2118	2188	5120	5122
NORTH AMERICAN (LUMINOUS)	1532		+	+	+	+	+	+			
	2190	+					+	+			
	2191	+			+	±					
	5105	+		+		+				+	
	6004	+		±	+						+
EUROPEAN (NON-LUMINOUS)	1667	+	+					+	+	+	+
	2118	+	+					+	+	+	+
	2188							+	+	+	+
	5120				+			+	+	+	+
	5122					+		+	+	+	+

FIG. 9. Table showing the results of pairing, in all possible combinations, series of monosporous mycelia from different collections of the American and European forms of *Panus stypticus*.

TABLE III

THE RESULTS OF SERIES OF PAIRINGS BETWEEN MONOSPOROUS CULTURES FROM DIFFERENT COLLECTIONS OF *Panus stypticus*

Culture number	Age when paired	Paired with culture number	Age when paired	Number of pairings	Clamp connections	
					Present	Absent
<i>American</i> × <i>American</i> 1532	1 yr. 147 days	2190	160 days	1	1	0
	5 yr. 280 days	2191	4 yr. 293 days	12	11	1
	5 yr. 282 days	5105	2 yr. 23 days	12	12	0
	5 yr. 280 days	6004	1 yr. 63 days	12	12	0
2191	4 yr. 295 days	5105	2 yr. 23 days	16	16	0
	4 yr. 293 days	6004	1 yr. 63 days	16	14	2
5105	2 yr. 20 days	6004	1 yr. 62 days	16	16	0
<i>European</i> × <i>European</i> 1667B	5 yr. 198 days	2118A	5 yr. 21 days	4	4	0
	5 yr. 198 days	2118B	5 yr. 21 days	12	12	0
	5 yr. 198 days	2188A	4 yr. 326 days	12	12	0
	5 yr. 198 days	5120	2 yr. 38 days	8	8	0
	5 yr. 198 days	5122	2 yr. 38 days	16	16	0
2118B	5 yr. 83 days	2188A	5 yr. 5 days	12	12	0
	5 yr. 83 days	2188B	5 yr. 5 days	4	4	0
	5 yr. 83 days	5120	2 yr. 102 days	4	4	0
	5 yr. 83 days	5122	2 yr. 102 days	16	16	0
2188A	5 yr. 5 days	5120	2 yr. 102 days	3	3	0
	5 yr. 5 days	5122	2 yr. 102 days	12	12	0
2188B	5 yr. 5 days	5120	2 yr. 102 days	1	1	0
	5 yr. 5 days	5122	2 yr. 102 days	4	4	0
5120	2 yr. 102 days	5122	2 yr. 102 days	4	4	0
<i>American</i> × <i>European</i> 1532	55 days	1667A	About 185 days	16	16	0
	1 yr. 303 days	1667B	1 yr. 237 days	1	1	0
	1 yr. 170 days	2118B	229 days	1	1	0
2190	162 days	1667B	1 yr. 75 days	1	1	0
	160 days	2118B	229 days	1	1	0
5105B	125 days	5120	118 days	56	56	0
6004	333 days	5122	1 yr. 285 days	64	64	0

sporous mycelia of two different collections. The signs used are as follows:

(+) to indicate that clamp connections have been found in every pairing of the series.

(+.) to indicate that clamp connections have been found in every pairing of the series but one.

(±) to indicate that clamp connections have been found in the majority of the pairings of the series.

Where no sign appears in the square, the series of pairings has not been made.

The results of 336 pairings have shown that, with three exceptions, complete fertility exists between the monosporous mycelia of all the collections paired, whatever their origin or host. The three exceptions are to be found in two series of pairings between monosporous mycelia of the American collections, namely 2191 \times 6004, where out of a series of 16 pairings two failed to produce clamp connections, and 1532 \times 2191 where in one pairing out of 12 no clamp connections were formed.

Inheritance of Luminosity

When it was found that monosporous mycelia of the luminous and non-luminous forms of *P. stypticus* were interfertile, an investigation into the problem suggested by Buller (6) as to whether or not luminosity in this fungus is an inherited character and, if inherited, the manner in which inheritance occurs was undertaken.

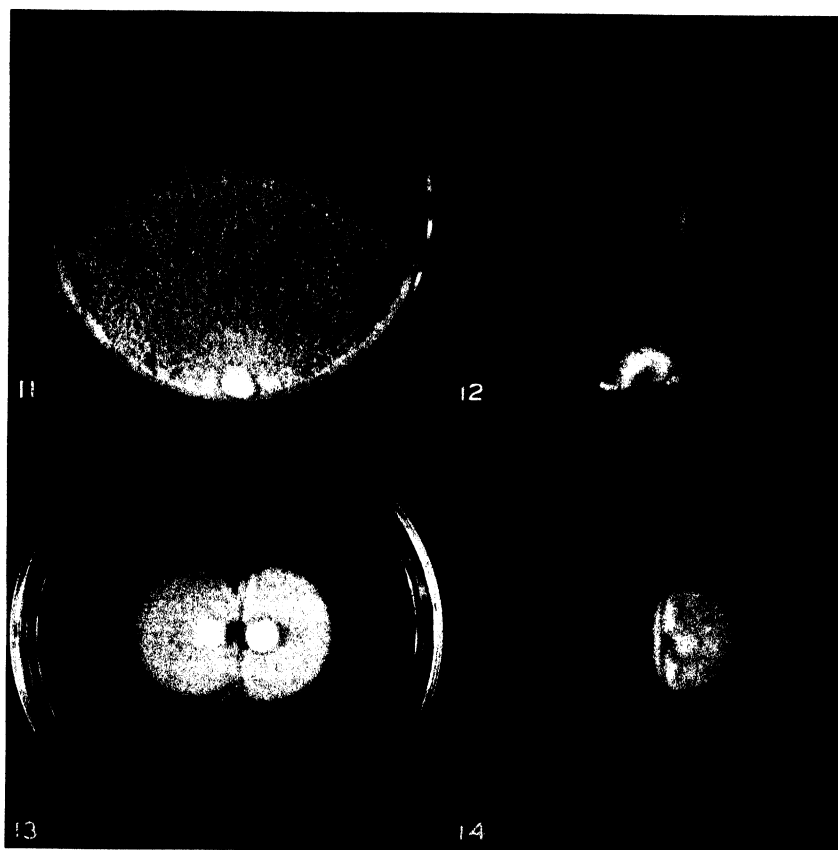
As a preliminary step, the diploid mycelium produced by the union of monosporous mycelia of the luminous American and the non-luminous European forms was recovered from the pairings listed below and examined for luminosity. The diploid mycelium from every pairing was found to be luminous.

Luminous		Non-luminous		Luminous		Non-luminous
1532-14	\times	1667B-7		5105B-6	\times	5120-9
1532-14	\times	2118B-4		5105B-8	\times	5120-9
2190-3	\times	1667B-7		5105B-8	\times	5120-20
2190-3	\times	2118B-4		5105B-16	\times	5120-20
5105B-1	\times	5120-1		5105B-18	\times	5120-12

Subsequently, two monosporous cultures were selected from each of the interfertility groups formed by the monosporous mycelia of the luminous American collection No. 6004 and the non-luminous European collection No. 5122 (Figs. 6 and 7). The eight monosporous mycelia of the luminous form were paired in all possible combinations with the eight monosporous mycelia of the non-luminous form (Fig. 10). Clamp connections were found

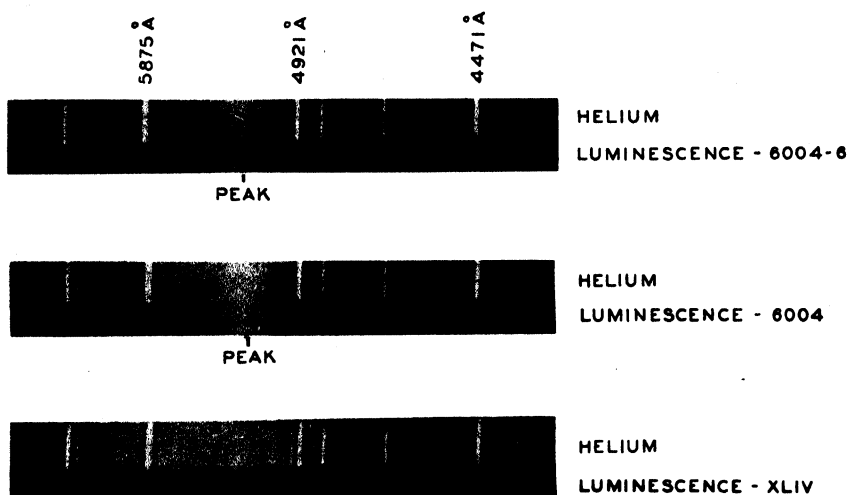
		5122							
		AB'	aB'	AB'	aB'	AB'	aB'	AB'	aB'
		1	19	12	25	23	26	18	20
6004	AB	12	+	+	+	+	+	+	+
	AB	15	+	+	+	+	+	+	+
	aB	22	+	+	+	+	+	+	+
	aB	27	+	+	+	+	+	+	+
	AB	6	+	+	+	+	+	+	+
	AB	9	+	+	+	+	+	+	+
	aB	10	+	+	+	+	+	+	+
	aB	26	+	+	+	+	+	+	+

FIG. 10. Table showing the results of pairing, in all possible combinations, eight monosporous mycelia from a single fruit body of the luminous American collection of *Panus stypticus*, No. 6004, with eight monosporous mycelia from a single fruit body of the non-luminous European collection, No. 5122.



Panus stypticus cultures grown on 1.25% malt agar. $\times 3$ approximately. FIG. 11. Diploid mycelium, four weeks old, from a pairing between a haplont, No. 6004-6, of the luminous American form and another haplont, No. 5122-12, of the non-luminous European form, photographed by reflected light. FIG. 12. The same culture as Fig. 11 photographed by its own light. FIG. 13. A two-weeks-old pairing between a non-luminous haplont, No. 5122-20, on the left, and a luminous haplont, No. 6004-6, on the right, photographed by reflected light. FIG. 14. The same pairing as Fig. 13 photographed by its own light. Note the luminosity of the haplont, No. 6004-6, and of the diploid mycelium formed by the union of the two haplonts in the area between the two inocula.

PLATE IV



15

FIG. 15. Spectra emitted by three cultures of *Panus stypticus* with helium comparison spectra. 6004-6: a monosporous culture and 6004: a polysporous culture of the luminous form of North American origin, XLIV: a diploid culture derived from the pairing of monosporous cultures of the luminous American form and the non-luminous European form, 6004-6 \times 5122-20.

in each of the 64 pairings and the diploid mycelium isolated from all the pairings was found to be luminous.

Photographs of one of these pairings, 6004-6 \times 5120-20, taken by reflected light and in the dark by its own light are given in Plate III. The luminosity of the diploid mycelium extending from the line of union between the two haplonts to the inoculum of the non-luminous haplont is clearly shown in the photograph taken in the dark.

The diploid mycelium produced by the pairings was isolated by transferring a very small piece of the mycelium from between the two inocula to a fresh malt agar slant and transferring this culture again several times. In 13 pairings, however, in order to avoid any possibility of the haploid mycelium being carried over, a minute inoculum from the diploid culture was placed on a malt agar plate and, as soon as a few hyphae had grown down into the agar, the inoculum was removed and a single hypha in the agar below was cut out with a fine needle, under the low power of a compound microscope, and transferred to a test-tube of malt agar. Isolation of single hyphae from the margin of a culture was found to be impossible as the individual hyphae there grew very close together.

Three of the 13 cultures, each derived from a single diploid hypha, namely XXXIX (6004-6 \times 5122-12), XLIV (6004-6 \times 5122-20), and XLV (6004-9 \times 5122-1), were grown on sections of *Betula papyrifera* branches, 6.5 to 9 cm. in diameter and 16 to 30 cm. long, which had the bark and part of the wood removed from one side. The sections were placed on sphagnum moss in a wide-mouthed jar, a little water added, the mouth of the jar covered with absorbent cotton, and the whole sterilized at 15 lb. steam pressure for one-half hour. The cultures were kept for five months in diffuse light in the laboratory and then from November until April were placed open under a bell jar in diffuse light in a greenhouse.

In March, 10 months after inoculation, two of the cultures, Nos. XXXIX and XLIV, had produced numerous rudimentary fruit bodies. Three of the fruit bodies of culture No. XLIV matured and shed spores (Plate I). When examined in the dark the gills were found to be brightly luminous. Five hundred and seven germinating spores from one of the three hybrid fruit bodies were isolated. Of these sporelings, 365 continued to grow and were examined for luminosity 27 and 54 days after isolation. Of the 365 cultures 198 were found to be luminous and 187 non-luminous, a ratio roughly of 1 : 1.

Nine of the luminous and 11 of the non-luminous monosporous cultures from the hybrid fruit bodies were paired together in all possible combinations with the results given in the table in Fig. 16. In this table the non-luminous cultures are marked by a line under the number of the culture. The results of the pairings show that the series of cultures is tetrapolar with both luminous and non-luminous cultures in each of the four interfertility groups.

It has been found (Hanna (19)) that the interfertility factors of the monosporous mycelia from a first generation fruit body may be determined by

		Ab						aB'					ab					AB'			
		1	4	11	20	22	24	7	14	16	17	18	21	2	8	10	12	13	15	3	19
Ab	1	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	11	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	20	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	22	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	24	-	-	-	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
aB'	7	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	14	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	16	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	17	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	18	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	21	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ab	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
AB'	15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	3	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-
	19	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-

FIG. 16. Table showing the results of pairing, in all possible combinations, a series of 20 monosporous mycelia from a single hybrid fruit body, No. XLIV, produced by the diploid mycelium from a pairing between a luminous and a non-luminous monosporous mycelium of *Panus stypticus* (6004-6 \times 5122-20).

backcrossing the F_1 mycelia with the mycelia of all four interfertility groups of both parents, since the interfertility factors of the parent mycelia are known and the monosporous mycelia from the F_1 fruit body pair only with those parent mycelia with which they have no common factor. In order, therefore, to determine the interfertility factors that should be assigned to each of the four groups of XLIV monosporous mycelia shown in Fig. 16, mycelia from each of the four groups were paired with each of the four groups of both parents. The results of these pairings are given in Fig. 17 where the interfertility factors for the monosporous mycelia of the parent collection, No. 6004, are designated as AB , ab , Ab , and aB and for the monosporous mycelia of the other parent collection, No. 5122, as $A'B'$, $a'b'$, $A'b'$, and $a'B'$.

Since the two monosporous mycelia which gave rise to the hybrid fruit body, No. XLIV, had the interfertility factors Ab and $a'B'$ (6004-6 \times 5122-20), the monosporous mycelia isolated from this fruit body would, if reduction division took place in the usual way, be expected to have the factors Ab , $a'B'$, AB' , and $a'b$, and to be compatible with the parent mycelia as follows:

<i>Ab</i> mycelia with	<i>aB</i> , <i>A'B'</i> , <i>a'b'</i> , <i>A'b'</i> , <i>a'B'</i>	mycelia
<i>a'B'</i> mycelia with <i>AB</i> , <i>ab</i> , <i>Ab</i> , <i>aB</i> ,	<i>A'b'</i>	mycelia
<i>AB'</i> mycelia with <i>ab</i> , <i>aB</i> ,	<i>a'b'</i> , <i>A'b'</i>	mycelia
<i>a'b</i> mycelia with <i>AB</i> ,	<i>aB</i> , <i>A'B'</i> , <i>A'b'</i>	mycelia

In the series of pairings shown in Fig. 17, however, not all the mycelia of one group of monosporous mycelia from the hybrid fruit body reacted in the same way when paired with the parent mycelia, and it was not clear from the pairings which of the pairs of factors should be assigned to the different groups. All of the pairings, therefore, were repeated and many were made a third

[illegible]

FIGS. 17 to 21. *Panus stypticus*. FIGS. 17 to 19. Tables showing the results of pairing 13 monosporous mycelia of the hybrid fruit body, No. XLIV, with two monosporous mycelia from each of the four groups of both parents. FIG. 20. A composite table of the results given in Figs. 17 to 19. FIG. 21. Ideal picture of the results expected when the actual results of the pairings are taken into consideration.

time. The results of these additional pairings are shown in Figs. 18 and 19. In Fig. 20 a composite table is given of all three series of pairings.

The results of these series of pairings indicate that monosporous mycelia Nos. 1, 4, 20, and 22 of the fruit body XLIV have the interfertility factors Ab , since, with the exception of two pairings, they are compatible with the parent mycelia having the factors aB , $A'B'$, $a'b'$, and $a'B'$, although some fertile pairings have been found between these mycelia and monosporous mycelia of each of the other groups. Likewise XLIV monosporous mycelia Nos. 7, 14, and 16 appear to have the interfertility factors $a'B'$, although three fertile pairings have occurred between two of these mycelia and parent mycelia bearing the factors $A'B'$.

The remaining two groups of monosporous mycelia, Nos. 3 and 19 and Nos. 2, 8, 13, and 15, both of which had one factor from each parent (AB' and $a'b$), when paired with the No. 6004 monosporous mycelia, appear to have the factors AB' and $a'b$ respectively, although XLIV-3 and XLIV-19 with the factors AB' have paired with 6004-6 with the factors Ab . These same two groups of mycelia, if assigned the factors AB' and $a'b$ respectively as suggested by their reactions with the No. 6004 monospores, show many compatible pairings between mycelia with a common interfertility factor and incompatible pairings between mycelia where no common factor is present when they are paired with the No. 5122 monosporous mycelia, and would seem to have the factors $a'b$ and AB' respectively rather than the factors AB' and $a'b$. As, however, the pairings of these monospores with the No. 6004 monospores gave clearer results, these two groups of F_2 monosporous mycelia have been assigned the factors AB' and $a'b$ respectively in Figs. 16 to 20.

In the whole series many compatible pairings have taken place between monosporous mycelia that have a common interfertility factor. They have occurred usually once or sometimes twice in a succession of three pairings of the same two mycelia, showing an instability or weakness of reaction not present in the majority of the compatible pairings in the series where no common factor was present. The clamp connections formed in these pairings were true not false clamp connections as Quintanilha (44) and Biggs (4) have found in illegitimate pairings in some species.

Quintanilha (43) and others have found that "illegitimate" pairings such as these, that is fertile pairings between haploid mycelia having a common interfertility factor, occur more frequently in some tetrapolar species between haploid mycelia that have one pair of factors in common rather than the other pair, that is when the B or b factors rather than the A or a factors are alike or vice versa depending on which factors are assigned to the group. In the series of pairings described here, the illegitimate pairings are not confined to mycelia that have one pair of factors in common but have occurred when any one of the four interfertility factors A , b , a' , or B' , have been held in common:

Illegitimate pairing	Common factor	Illegitimate pairings	
		No.	%
$Ab \times AB$	A	5	62.5
$Ab \times ab$	b	1	12.5
$Ab \times Ab$	A and b	3	37.5
$aB' \times A'B'$	B'	3	50
$AB \times Ab$	A	2	50
$AB' \times A'B'$	B'	4	100
$a'b \times a'b'$	a'	7	87.5

In three of the pairings both interfertility factors, $Ab \times Ab$, were held in common by the paired mycelia. It may be noted also that the haploid mycelia of the hybrid fruit body XLIV were paired together in all possible combinations, Fig. 16, the haploid mycelia XLIV-20 \times XLIV-24, having these same two factors, $Ab \times Ab$, also paired with each other.

Quintanilha (43) found that illegitimate pairings in *Coprinus fimentarius* occurred more frequently when the mycelia paired were young and often disappeared with the ageing of the mycelia. In this series, however, the mycelia were fully mature: 6004 haplonts—2 yr., 338 days; 5122 haplonts—3 yr., 228 days; XLIV haplonts—169 days.

It has been suggested by Kniep (31) that the ability of two monosporous mycelia to pair is dependent upon certain quantitative differences in the fertility factors, differences that have a maximum and a minimum beyond which pairing does not take place. When, for example, in certain individuals the difference between the B factors is sufficiently great, B (or b) individuals can pair with other B (or b) individuals if the equality of the A factors does not stand in the way. If, on the other hand, a difference approaching a minimum occurs, a slight variation means that the minimum difference for copulation is not reached and sterility results.

Whether the irregularity in the behaviour of the monosporous mycelia of the hybrid fruit body XLIV when paired with the mycelia of the parent generation is the result of quantitative changes in the factors such as this or whether it is brought about by mutations or other chromosome changes has not been determined.

Discussion

Since the diploid mycelium and hybrid fruit bodies from a cross between the luminous and non-luminous forms of *P. stypticus* are luminous and the haploid mycelia derived from such a cross may be separated into two approximately equal luminous and non-luminous groups, it must be concluded that the factor for luminosity in this fungus is an inherited one and that luminosity is dominant over non-luminosity. Since, also, both luminous and non-luminous mycelia are to be found in all four interfertility groups of haploid mycelia of the F_1 hybrid fruit body, it is evident that the factor for luminosity and its allelomorph form all possible combinations with the interfertility factors.

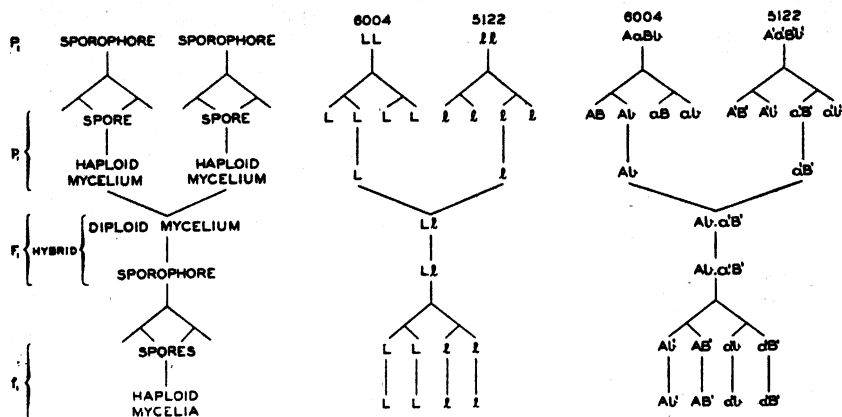


FIG. 22. Diagrams showing the inheritance in *Panus stypticus* of the luminosity and interfertility factors in a cross between haploid mycelia of a luminous American collection, No. 6004, and a non-luminous European collection, No. 5122.

The method of inheritance of the luminosity factor, suggested by these experiments, is presented graphically in Fig. 22 where the factor for luminosity is represented by the letter L and its allelomorph, non-luminosity, by the letter l . The sporophore of the luminous American form or P_1 generation possesses the factors LL and the sporophore of the non-luminous European form, their allelomorphs ll . After reduction division of the fusion nucleus in the basidia of the P_1 generation, each of the spores and haploid mycelia of the luminous sporophore carries one factor for luminosity, L , and each of the spores and haploid mycelia of the non-luminous sporophore its allelomorph l . The diploid mycelium resulting from the union of luminous and non-luminous haploid mycelia and the sporophores produced by such diploid mycelia possess the two factors Ll . Upon reduction division the luminosity factors are divided equally among the four spores and the ratio of luminous to non-luminous haploid mycelia in the f_1 generation is 1 : 1, which is the Mendelian ratio of a dominant to its allelomorph in the gametes of the f_1 generation.¹

The gametophytic and sporophytic generations characteristic of the life history of most plants are distinguished from each other by the number of chromosomes present in the nuclei of each generation, the former having the single or haploid number of chromosomes and the latter the double or diploid number. In the heterothallic Basidiomycetes the basidiospores and the monosporous mycelia arising from them, having nuclei with the haploid number of chromosomes, constitute the haploid or gametophytic phase in the life history of these fungi. When two compatible haploid mycelia unite, a diploid mycelium is formed in which the haploid nuclei are associated in pairs within the cells. The paired nuclei divide conjugately during the devel-

¹ In assigning symbols to the different generations here and in Fig. 22 the scheme followed by Allen (1, 2) is used, namely P_1 , F_1 , F_2 etc. for the diploid generations and p_1 , f_1 , f_2 etc. for the haploid generations.

opment of the mycelium and fruit bodies until in the basidium they unite to form a single fusion nucleus. The binucleate mycelium and fruit bodies and the basidia constitute the diploid or sporophytic phase.

The alternation of haploid and diploid phases in the life history of the Basidiomycetes may be compared to the alternation of gametophytic and sporophytic generations in the higher plants, with this difference: in the higher plants the nuclei of the gametes fuse when they come together within a single cell whereas in the heterothallic Basidiomycetes the two haploid nuclei remain associated together without union throughout an appreciable stage of the life cycle. Their fusion in the basidium may be considered a delayed sexual fusion.

It has been shown by the work of Zattler, Gilmore, Dickson, Nobles, and Quintanilha and Balle already reviewed, and by the study of the inheritance of uredospore colour, pustule type, and pathogenicity in the Uredinales and the inheritance of the general morphology and consistency of the sori, the colour of the peridia of the sori, the degree to which the host plant may be stunted, and pathogenicity in the Ustilaginales that the genes governing the inheritance of different characters behave in the dikaryotic mycelium and fruit bodies of the Basidiomycetes, although contained within two separate nuclei, as they do in higher plants where they have come together within a single nuclear membrane.

Dodge (14) writes that a cell may be considered diploid only when its nucleus contains the double number of chromosomes and hence cells containing two haploid nuclei, even though of opposite "sex", are not diploid but rather dikaryotic haploid partnerships and that the true diploid in the Basidiomycetes is to be found only in the basidium where the nucleus, after fusion has taken place, contains the double number of chromosomes. In dealing with the genetics of fungi possessing such dikaryons he considers the dikaryotic mycelium resulting from the union of the two haploid mycelia of different genetic origin not as true hybrids but rather as graft hybrids or chimaeras and that since only diploid nuclei can be heterozygous any apparent dominance in such mycelia is a false one.

Johnson and Newton (25), Zattler (55), Stakman, Levine, and Cotter (49), and Buller (7, 8) have expressed the opposite view that since the two sets of genes within a diploid cell have been shown to act as though contained within a single nuclear membrane the dikaryotic mycelium and fruit bodies are truly diploid and are true hybrids.

The evidence of dominance of the factor for luminosity in the diploid mycelium and fruit bodies produced by pairing luminous and non-luminous haploid mycelia of *Panus stypticus* adds further evidence in support of the latter authors' views.

Acknowledgments

These investigations were carried out at the Department of Botany, University of Toronto, and at the Division of Botany and Plant Pathology, Department of Agriculture, Ottawa. The writer wishes to express her

appreciation and gratitude to Professor H. S. Jackson for his advice and criticism during the course of the work, and to thank Dr. H. T. Güssow, Dominion Botanist, for his unfailing interest and for supplying the European material, Dr. A. H. R. Buller for his interest in the problem which he suggested in his *Researches on Fungi*, Volume III, and for supplying three of the American collections, Dr. J. W. MacArthur for reading and criticizing the manuscript, and Dr. Irene Mounce for her helpful suggestions. She wishes also to thank Dr. L. E. Howlett of the Division of Physics and Engineering of the National Research Council of Canada for his kindness in taking the photographs of the spectra of the cultures.

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INDIVIDUAL PLOT STUDIES OF VARIATION IN NUMBERS OF BACTERIA IN SOIL

I. RESPONSE TO CROPPING¹

BY NORMAN JAMES² AND MARJORIE L. SUTHERLAND³

Abstract

Studies on the relation of cropping to numbers of bacteria in soil were carried on in an experiment designed for statistical control. Plots were cropped as follows: fallow, three; wheat, three; barley, two; sugar beets, two; soybeans, two; and corn, two. The estimate on each plot is based on counts from 24 plates. A series of from 17 to 20 such estimates, made at weekly intervals from each plot, is available for the analysis. Moisture data are available also. The effect of cropping on numbers of bacteria is shown for each plot as the multiple regression of bacteria on days and moisture reduced to two dimensions by the elimination of moisture.

Bacteria respond to a growing crop. This response is similar in replicate plots of one treatment; but is markedly different with different crops.

Introduction

In a series of papers published recently (2, 3, 7) the authors considered some of the basic weaknesses of the plating technique for estimating numbers of bacteria in soil, and showed how the procedure can be improved to yield an estimate reproducible within narrower limits than had been possible hitherto. Some of the changes are mechanical. They relate to the length of time the sample is held in the laboratory, the proportion of soil to water in the initial dilution, and the method and time of mixing at various stages in the procedure. Another change that is of greater importance (6) in producing a precise estimate of the population in a plot is based on the fact that the technique involves a series of samplings from biological populations varying at random at each sampling step. A minimum of two samples at each step is required to obtain an estimate of error. In another paper (4) differences in the number of colonies on plates were shown to have a marked influence on the estimate of the population in the plot. This effect may be studied by preparing and plating from two or more dilutions of different strengths from one initial dilution. The authors also (5) presented evidence that fluctuations in numbers of bacteria in the soil of a fallow plot may be

¹ Manuscript received in original form March 16, 1942, and as revised, June 11, 1942.

Contribution from the Department of Bacteriology and Animal Pathology, The University of Manitoba, Winnipeg, Man., with financial assistance from the National Research Council of Canada.

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attributed largely to changes in moisture and dates of sampling. The latest finding was dependent upon estimates, each based on counts from 240 to 324 plates and representing many replications of samples, subsamples, and dilutions.

The work reported in this series was designed for two purposes: (1) to determine whether estimates made at weekly intervals from counts on 24 plates would show changes in numbers of bacteria during the growing season in plots supporting different crops, and (2) to obtain additional information on the errors associated with each sampling step in the procedure.

This paper deals with the first point only. Other studies based on the same data will be presented later.

Experimental

During the summer of 1940 a series of 14 one-hundredth acre plots was laid out on a portion of the University farm that had been fallow the previous year. These were randomized and cropped as follows; fallow, three; wheat, three; barley, two; sugar beets, two; soybeans, two; and corn, two plots, respectively. In order to lessen the labour on any one day, the plots were divided into two groups determined at random each week. One group was sampled and plated on one day, and the other on another day in the same week. This continued from May 22 to October 1. It provided data for a series of estimates from each plot during the crop season. Each estimate was based on counts from 24 plates, and the design provided for information on the errors at each step in the procedure. Duplicate samples were obtained on each date a given plot was sampled. Each was a composite of six cores of the upper six inches of soil, taken from points determined at random at each sampling. The soil was grated mechanically by being passed through a four mesh per inch wire screen under slight pressure, and mixed in a revolving drum for 10 min. Each of two 50 gm. subsamples of moist soil from each sample was suspended in 2500 ml. of low count tap water and shaken for 10 min. on a to-and-fro mechanical shaker. A 1 ml. transfer was made during agitation of the suspension to a sterile 99 ml. blank. The second dilution was shaken by hand 25 times. From this 1 ml. transfers were made to sterile 79, 39, and 25.67 ml. dilution blanks, resulting in final dilutions of 1 : 400,000, 1 : 200,000, and 1 : 133,000, respectively. These dilutions were chosen in order, theoretically, to obtain counts in the proportions of 1 : 2 : 3. Each dilution was plated in duplicate in Waksman's sodium albuminate agar and incubated for six days at 25 to 27° C. A moisture determination was made on a 50 gm. portion of each sample obtained after the mechanical mixing, and each count was converted to provide an estimate per gram of oven-dried soil. These individual plate estimates were used in the analysis to obtain information on the errors of the laboratory technique, and the average of 24 such estimates to provide the plot estimate on any date of sampling.

Some of the plots were sampled on 20 different dates, while others because of unfavourable weather were sampled 19, and still others only 18 times.

Data from one plot on 20 different dates give 19 degrees of freedom for estimating the variance for between dates. One of these represents the portion of the variance for the multiple correlation of bacteria with moisture and time that is independent of time. Another, and in some cases two, represents the portion that is independent of moisture. The remaining degrees of freedom represent the unexplained portion of the variance for between dates. This is the error for testing the significance of the variances for moisture and time. It is designated Error 1. On the average it will not be less than the largest of the variances for laboratory and field sampling effects which in this experiment is the variance for within pairs of samples from a plot.

Each plot estimate on any date is the average of 24 individual plate estimates. This gives 23 degrees of freedom for estimating the within date variance, 14 of which are assigned to certain laboratory and field sampling effects and their interactions according to the design of the experiment. The remaining nine degrees of freedom represent the unexplained portion of this variance. It is designated Error 2. This error is used to test the significance of the variances for laboratory and field sampling effects as well as for testing the significance of Error 1. The data from a plot sampled on 20 different dates give a total of 460 degrees of freedom for estimating the variance for within dates. This variance is an essential part of the experimental design. It will be considered more fully in a subsequent paper in this series. The combined variances for between dates and within dates, accordingly, represent a grand total of 479 degrees of freedom. The form of the analysis follows.

Source of variance	Degrees of freedom	
Between dates	19	
Moisture	1	
Time	1	
Error 1	17	
	1 Unit	20 Units
Within dates	23	460
Samples	1	20
Subsamples	2	40
Dilutions	2	40
Dilutions \times samples	2	40
Dilutions \times subsamples	4	80
Plates	1	20
Dilutions \times plates	2	40
Error 2	9	180
Grand total		479

Changes in Numbers of Bacteria Associated with Cropping

The population in a given plot responds tremendously to changes in moisture and time. This has been demonstrated (5) with estimates based on counts from 240 to 324 plates per estimate. Accordingly, some method of analysis for removing the effects of moisture must be used if the effect of cropping on

numbers of bacteria is to be presented clearly. The moisture-population relationship is not a simple one. The moisture content of a plot affects the population directly. Moisture is influenced by a crop. Consequently, a crop indirectly affects the population by its effect on the moisture, apart from any direct effect it may have on the population. Obviously, the indirect effect of cropping on the population is not the same for different crops or at different stages in the development of a crop. Fig. 1 illustrates the differences in moisture between a fallow and a barley plot during the course of this experiment.

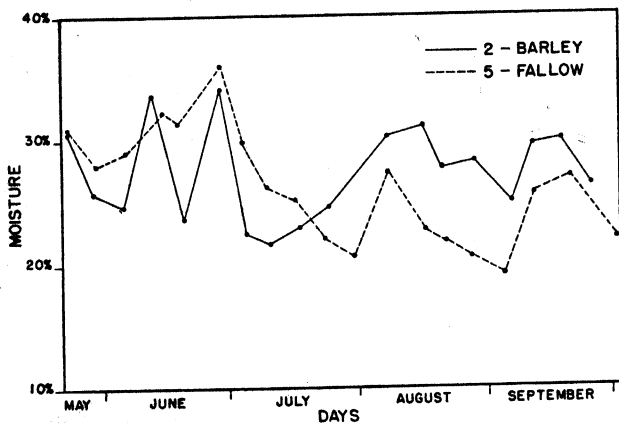


FIG. 1. Moisture in a fallow and a barley plot during the crop season.

In this study from 17 to 20 estimates per plot are available. Each is based on counts from 24 plates. Further, each represents a different date and a different stage in the development of a crop. Moisture data are available also.

The data for each plot were treated separately, since there is no valid reason for assuming that the level of the population in one plot is directly comparable to that in another and sufficient information on the response of bacteria in plots is not available to justify a detailed comparison of plot populations in this study. A multiple correlation of bacteria in millions, moisture in percentage, and time in days from the beginning of the experiment was calculated from the data for each plot. In corn and barley plot data the bacteria-time relationship is non-linear, and a significant portion of the total sum of squares is explained by including a fourth variable (days²) in the multiple correlation. In these cases where B represents number of bacteria, M , moisture in percentage, and D , number of days, the regression equation becomes:

$$\text{Estimated bacteria} = \bar{B} + b_{BM}(M - \bar{M}) + b_{BD}(D - \bar{D}) + b_{BD^2}(D^2 - \bar{D}^2)$$

Since the response of the population to changes in time is the main point of interest in this investigation, numbers of bacteria for each date of sampling were estimated from the multiple regression equation with moisture set at a

constant of 25%. This was repeated for each plot, giving regression lines that represent changes in numbers of bacteria during the crop season. These are shown in Fig. 2. A fallow plot has a time effect, but not a cropping one. Accordingly, it may be considered as a check plot in a study of the effect of cropping on numbers of bacteria.

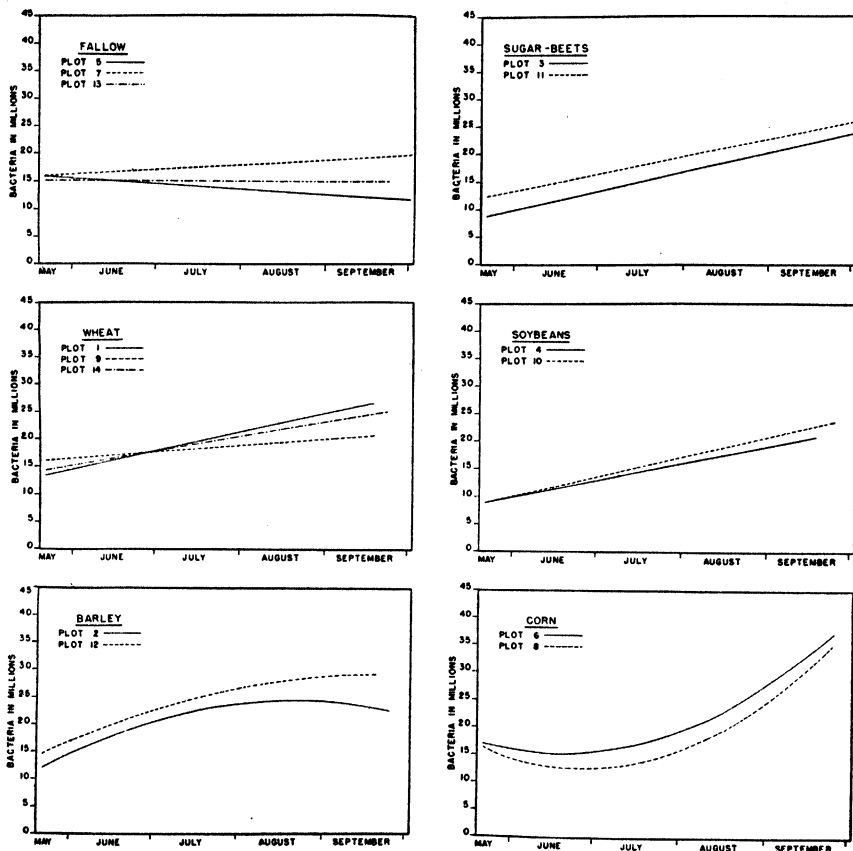


FIG. 2. Regressions of bacteria on time, with moisture constant at 25%, for each plot during the crop season.

In reality, correlation is a form of analysis of variance. The square of the correlation coefficient represents the percentage of the total sum of squares accounted for by the relationship. Accordingly, to avoid confusion in subsequent papers in this series, the correlation data for individual plots are expressed in terms of analysis of variance. The total sum of squares for between dates represents a portion explained by the multiple correlation of bacteria with moisture and time and an unexplained portion. Further, the sum of squares for the correlation consists of a portion related to moisture

that is independent of time and another related to time that is independent of moisture. These were calculated by the method given by Goulden (1, p. 214, Equation 10). These data appear in Table I.

TABLE I

Source of sum of squares	Sum of squares	Degrees of freedom	Sum of squares	Degrees of freedom	Sum of squares	Degrees of freedom
Fallow	Plot 5		Plot 7		Plot 13	
Moisture	2747.1168	1	7427.9760	1	2928.5400	1
Time	1399.7112	1				
Error 1	1185.6336	16	2478.5664	18	1466.5320	16
Wheat	Plot 1		Plot 9		Plot 14	
Moisture	6536.2080	1	2694.5736	1	6512.8872	1
Time	6706.0512	1	701.6040	1	2953.9176	1
Error 1	7890.8232	15	2156.3688	14	3550.8744	15
Barley	Plot 2		Plot 12			
Moisture	3375.0840	1	5976.8640	1		
Time	5978.7048	2	8913.6336	2		
Error 1	6938.2944	14	6630.1488	14		
Sugar beets	Plot 3		Plot 11			
Moisture	6088.3128	1	5618.0928	1		
Time	1801.3392	1	2407.1544	1		
Error 1	5218.2648	15	3889.1808	16		
Soybeans	Plot 4		Plot 10			
Moisture	9081.4440	1	11518.9464	1		
Time	17.1024	1	3024.9696	1		
Error 1	1787.1336	14	4598.0832	16		
Corn	Plot 6		Plot 8			
Moisture	11975.8464	1	4755.9264	1		
Time	15538.9656	2	17204.1264	2		
Error 1	7386.5568	15	3871.1400	14		

In order to show more clearly the effects of moisture and time on numbers of bacteria in the various plots the sums of squares and degrees of freedom for Error 1, moisture, and time were combined for plots receiving one treatment. New variances were calculated. These are shown in Fig. 3.

Moisture and time variances may be compared with Error 1. The portion of the variance associated with time and independent of moisture is small in the data from fallow plots. Cropped plot data show a time response that varies from small in soybeans to moderate in barley and large in corn. This is fair evidence that the growing crop has a definite influence on numbers of bacteria in the soil. This is in addition to the response the population makes to moisture.

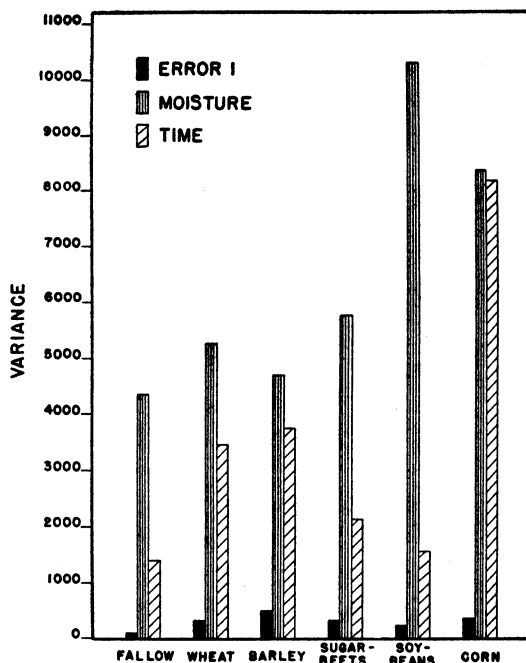


FIG. 3. Variances for Error 1, the correlation of bacteria and moisture independent of time, and the correlation of bacteria and time independent of moisture. The data for replicate plots are combined for each crop.

Discussion

The data presented indicate that the plate count method, as carried out in this experiment, is highly sensitive. This sensitivity is due to the reduction of Error 1, which results from minimizing the variances for within date effects by revisions in the field and laboratory sampling procedures, and further, to the duplication of samples at each step to balance for random sampling variations.

Precise estimates of the bacterial population, obtained by the revised method, should not be presented merely as a graph of numbers of bacteria against dates of sampling. There are so many environmental factors affecting the estimate on any date that such a simple presentation has little value in relation to any effect under consideration. In the data presented, the effect of moisture on numbers of bacteria in any plot was so great that the graph representing numbers of bacteria plotted against dates of sampling for the season gives a wrong impression of the effect of cropping on numbers of bacteria.

When the effect of moisture on numbers of bacteria is removed by statistical treatment there is a better chance of measuring the effects of additional factors on numbers of bacteria. In this study the second factor considered is cropping. It is only one of many that may affect the population.

The plate count method, in the revised form, is practical for experimental plot studies. It has been proven highly sensitive in demonstrating the response of bacteria to changes in moisture and to cropping. Undoubtedly, it will be equally valuable in similar studies dealing with response to differences in many chemical, physical, and biological factors in soil. The method, as used formerly, has been applied in several large scale investigations undertaken to show the relationships between various factors and numbers of bacteria. Samples were obtained from widely separated areas and, as a consequence, the amount of routine labour was great. Frequently results were inconsistent and inconclusive. On the basis of results in this study there is reason to expect that the revised procedure could be used in this type of experiment if the design were considered carefully. Otherwise the extreme sensitivity of bacteria to environment would cause wide variation among estimates. Lack of information on the response of this population to differences in environment would make it impossible to attribute this variation to anything but error. An unnecessarily large error might render the data useless for the purpose intended. This chance of failure may be reduced by designing the experiment to obtain information simultaneously on different factors that affect numbers of bacteria in soil. When data on many factors are available, much of the variation can be explained on the basis of response to specific effects and may be separated in the analysis. The error variance is reduced, thereby giving a more sensitive test of any effect under investigation.

Finally, the authors are confident that the plate method eventually will find general application in the problems of soil fertility, the control of soil-borne plant pathogens and soil classification. Its value in relation to these problems in large measure will depend on a recognition of the need for valid information on the many different sources of variation in laboratory and field sampling procedures and on a realization of the extreme sensitivity of bacteria to many environmental factors. Undoubtedly, progress will be made through the use of highly selective media in experiments designed for statistical control. Differences in the number of bacteria developing on one medium may indicate variations in a definite soil condition in about the same way that differences in colour of one pH indicator show variations in acidity within a narrow range of the pH scale.

Acknowledgments

The authors are pleased to express thanks to Dr. C. H. Goulden, Senior Agricultural Scientist, Dominion Rust Research Laboratory, Winnipeg, and Honorary Lecturer in Statistics, The University of Manitoba, for assistance in the statistical part of this study; to Mr. Robert Mitchell, The University of Manitoba, for preparing the charts; and to Mr. A. M. Brown, Assistant Plant Pathologist, of the Dominion Rust Research Laboratory, for photographing the illustrations.

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INDIVIDUAL PLOT STUDIES OF VARIATION IN NUMBERS OF BACTERIA IN SOIL

II. THE ERRORS OF THE PROCEDURE¹

BY NORMAN JAMES² AND MARJORIE L. SUTHERLAND³

Abstract

Data on the errors of the plate count method are presented. They are based on changes in numbers of bacteria during the crop season in plots supporting different crops. Duplicate samples were used at each step in the procedure. This provides information on variations associated with sampling, which contribute to the error of the plot estimate on any date.

A large portion of the differences among estimates from each plot made on different dates is explained by correlations among numbers of bacteria and changes in environmental factors. Obviously, a large error masks a small relationship.

This may be minimized by (1) careful sampling and the use of duplicates at each step in the procedure and (2) collecting data for correlating bacteria with changes in many environmental factors other than the one of chief interest in the investigation.

Introduction

Satisfactory plate count data from soil imply more than a carefully carried out laboratory routine. They represent results that can be interpreted in their true relation to an effect under investigation. This involves a design for the experiment based on certain definitely known facts and principles which may be listed briefly.

1. The soil complex lacks homogeneity. Consequently, estimates on two samples taken at points determined at random may differ more than would be expected on the basis of random sampling from a biological population.

2. The degree of dilution required to obtain a small enough portion of the original soil to provide countable numbers of colonies on plates makes necessary a series of sampling steps between the soil in the plot and the colonies on the plate. Each sampling step involves random sampling from a biological population and probably, as well, additional variation.

3. An estimate based on replicate samples taken at random is better than one from a single sample. This applies at each step in the procedure.

4. Replication of samples provides data for measuring the variation attributable to any sampling step.

5. Bacteria in soil are extremely sensitive to environmental changes. Consequently, information on many factors should be available from the plot on each sampling date. The responses to these may be segregated in the analysis.

¹ Manuscript received in original form March 16, 1942, and as revised, June 11, 1942.

Contribution from the Department of Bacteriology and Animal Pathology, The University of Manitoba, Winnipeg, Man., with financial assistance from the National Research Council of Canada.

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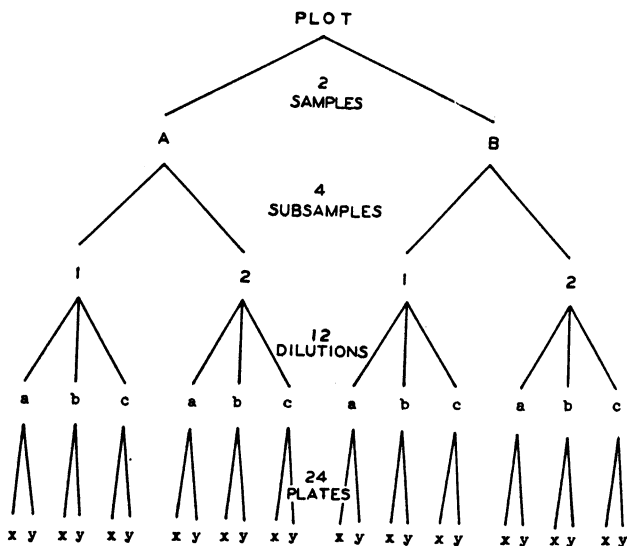
6. The portion of the difference between plot estimates made on different dates that cannot be assigned to any of the factors considered is the error used to test the significance of the response to any of them.

7. A small error gives a more sensitive test of an effect. Consequently, the plate count method of demonstrating the relation between numbers of bacteria in soil and any factor is made more sensitive by increasing the portion of the bacterial response that can be assigned to other specific factors.

This paper deals with the problem of errors in an experiment designed to show the effect of cropping on numbers of bacteria in soil. The data reviewed have been considered (4) in relation to bacterial response to cropping. Details as to crops, plots, sampling, and the laboratory procedure have been presented.

Design of the Experiment

The design of the experiment is considered in two parts. One part provides information on the various steps in the laboratory procedure and has to do with the reliability of the estimate for a plot on any date of sampling. It deals with the within date variance. It is illustrated below.



This 24 plate unit has 23 degrees of freedom for estimating the within date variance. These may be divided to give information on the following.

Source of variance	Degrees of freedom
Total	23
Samples	1
Subsamples (2×1)	2
Dilutions (4×2)	8
Plates (12×1)	12

There is no reason to expect a consistent difference between samples or between subsamples. Consequently, the interaction of samples and subsamples is not considered. The three dilutions represented different proportions of soil to water. There would be different numbers of colonies on plates. Probably this would introduce an effect related to the differences between the associative action among colonies on plates from one dilution and that among colonies on plates from a different dilution. Accordingly, the eight degrees of freedom for dilutions are split to show the variances for dilutions, the interaction of dilutions \times samples and the interaction of dilutions \times subsamples. The 12 pairs of plates were handled in a systematic way. Each pair was prepared, piled in the incubator, and counted in one order. The bacteria on pairs of plates might show response to this systematic procedure. Accordingly, 1 of the 12 degrees of freedom for plates is segregated to represent systematic order. Obviously, the interaction between the three dilutions and plates prepared in this order should be considered. This interaction accounts for two more degrees of freedom. The remaining degrees of freedom represent interactions not likely to be of particular interest in this study. They are grouped as the error for the 24 plate unit. The form of analysis follows.

Source of variance	Degrees of freedom	Source of variance	Degrees of freedom
Total	23	Dilutions \times subsamples	4
Samples	1	Plates	1
Subsamples	2	Plates \times dilutions	2
Dilutions	2	Error 2	9
Dilutions \times samples	2		

The other part of the design provides information on the bacterial response in 14 plots to moisture and to different crops as well as on Error 1. These represent between date variances. The method of handling the data to separate the variance for these effects has been considered in the previous paper.

The degrees of freedom for the entire experiment are shown in Table I. The variation in degrees of freedom from different plots is due to the fact that all plots were not sampled on the same number of dates. The degrees of freedom for within date effects and interactions represent those shown in the design for one plot estimate multiplied by the number of dates on which each plot was sampled.

Results

For simplicity in presentation and brevity, analysis of variance data are presented in Fig. 1 in what appear to be six graphs. Each graph represents a different crop treatment. The sources of variance are listed at arbitrary intervals along the X axis in approximately the reverse order to that in Table I. The mean squares for each source of variance are plotted on the Y axis on semilogarithmic paper and the points joined arbitrarily. This makes it possible to visualize and compare mean squares in the 1000's range from certain sources of variance along with others in the 10's range from different

TABLE I

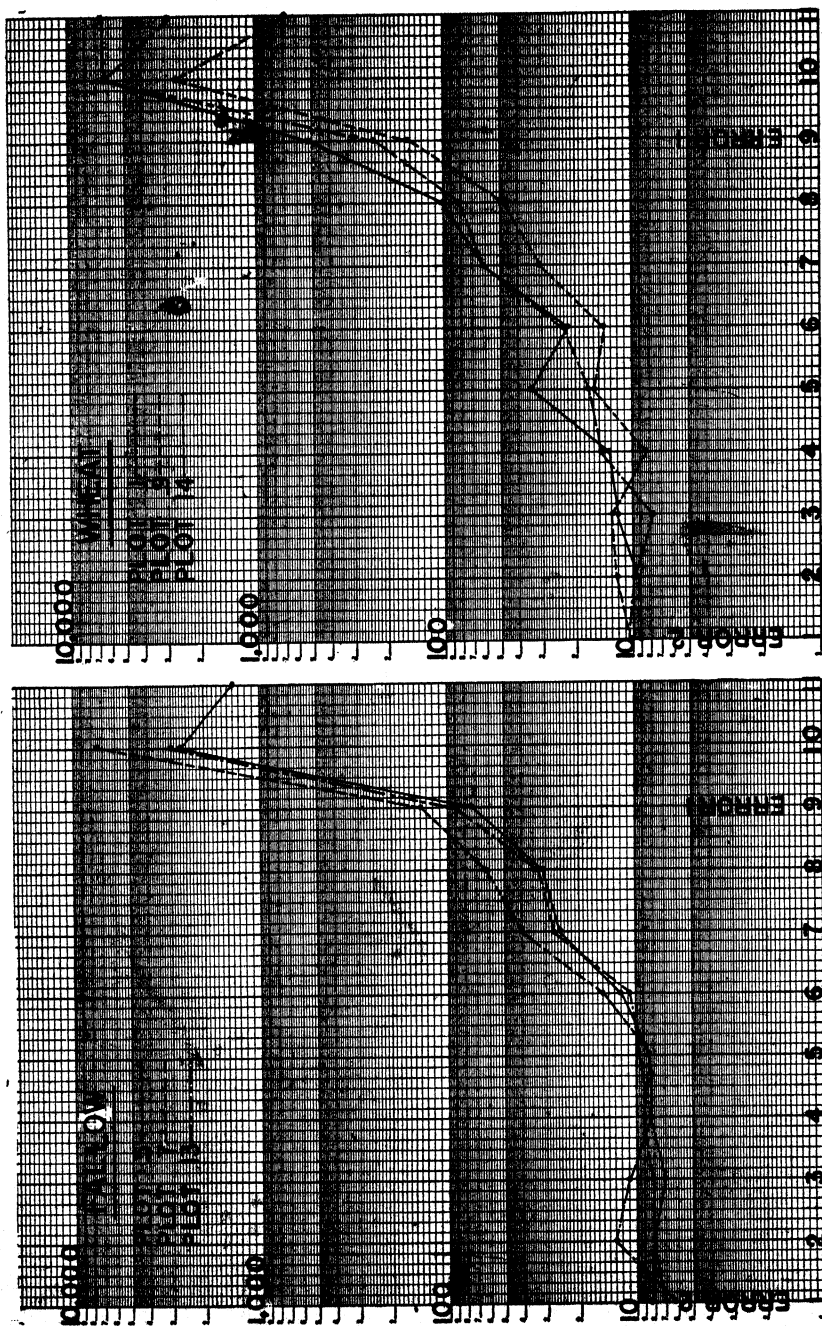
Source of variance	Crop							
	Fallow			Wheat			Barley	
	Plot							
	5	7	13	1	9	14	2	12
Between dates								
Total	18	19	17	17	16	17	17	17
Moisture	1	1	1	1	1	1	1	1
Time	1	—	—	1	1	1	2	2
Error 1	16	18	16	15	14	15	14	14
Within dates								
Total	437	460	391	414	391	391	414	391
Samples	19	20	17	18	17	17	18	17
Subsamples	38	40	34	36	34	34	36	34
Dilutions	38	40	34	36	34	34	36	34
Dilutions × samples	38	40	34	36	34	34	36	34
Dilutions × sub-samples	76	80	68	72	68	68	72	68
Plates	19	20	17	18	17	17	18	17
Plates × dilutions	38	40	34	36	34	34	36	34
Error 2	171	180	153	162	153	153	162	153

Source of variance	Crop					
	Sugar beets		Soybeans		Corn	
	Plot					
	3	11	4	10	6	8
Between dates						
Total	17	18	16	18	18	16
Moisture	1	1	1	1	1	1
Time	1	1	1	1	2	2
Error 1	15	16	14	16	15	13
Within dates						
Total	414	437	391	437	437	391
Samples	18	19	17	19	19	17
Subsamples	36	38	34	38	38	34
Dilutions	36	38	34	38	38	34
Dilutions × samples	36	38	34	38	38	34
Dilutions × sub-samples	72	76	68	76	76	68
Plates	18	19	17	19	19	17
Plates × dilutions	36	38	34	38	38	34
Error 2	162	171	153	171	171	153

sources. If tests of significance are desired approximate mean squares may be read directly from the graphs and degrees of freedom obtained from Table I. An error may be selected according to the principles of analysis of variance and *F* values calculated.

Key to Numbers on the X Axis of Fig. 1.

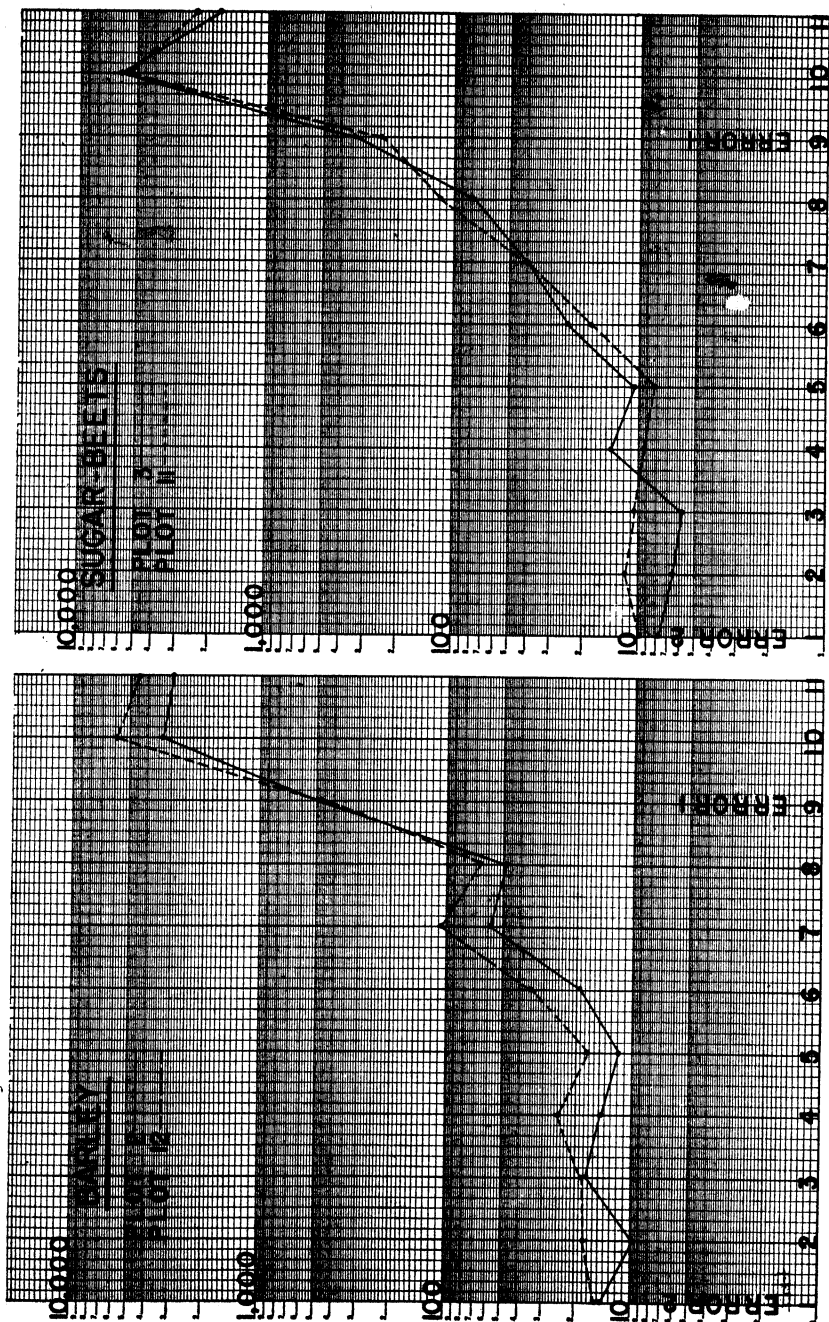
- | | | |
|---------------------------|------------------------|--------------|
| 1. Error 2 | 5. Dilutions × samples | 9. Error 1 |
| 2. Plates × dilutions | 6. Subsamples | 10. Moisture |
| 3. Plates | 7. Dilutions | 11. Time |
| 4. Dilutions × subsamples | 8. Samples | |



(b)

FIG. 1

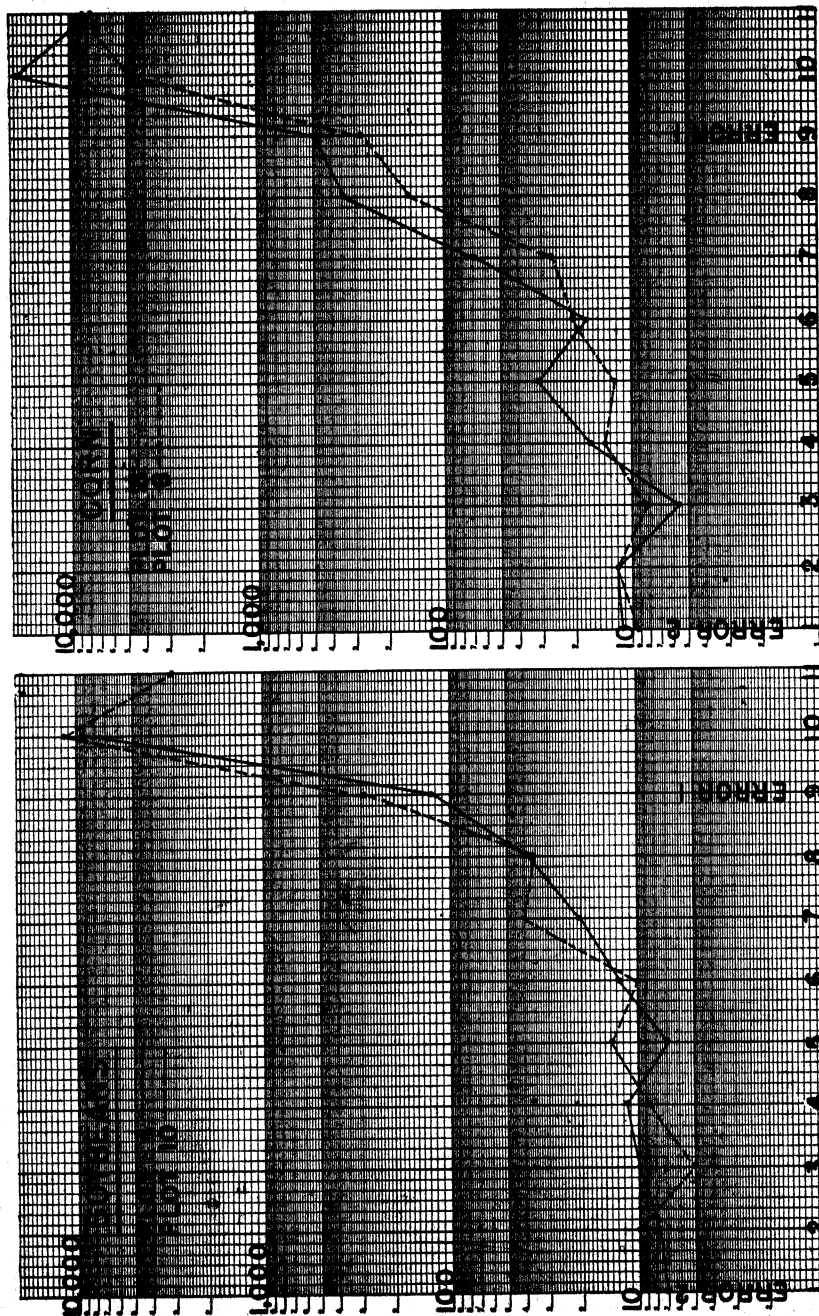
(a)



(d)

(c)

FIG. 1



(f)

FIG. 1

(e)

The graphical presentation of the data makes several points apparent.

1. The graphs are much alike. This is evidence that, in general, the mean squares for any source of variance are quite similar for all plots, even though they represent different crop treatments. Of course, the mean squares for time are exceptions.

2. The mean squares for each plot in any chart hold different levels, with little crossing of the lines connecting the points. This may be explained by the presence of populations differing in size in the different plots but having similar tendencies to vary in response to environmental conditions. This would give a proportionately greater mean square at each sampling step in the plot having a larger population. Or, it may be explained by the presence of populations made up of different proportions of various biological groups. One plot may contain a large proportion of a group that is sensitive to changes in environment, while in another plot this group may represent only a small portion of the population counted.

3. The first six variances, Error 2 to subsamples, in general, are quite similar in any chart. They hold different levels in different crops.

4. Theoretically, the number of colonies per plate should vary as 1 : 2 : 3 and the variance for dilutions should be little greater than Error 2. In these data the mean squares for dilutions are several times as large. This was expected on the basis of previous work (1). Accordingly, the variance associated with this difference in number of colonies per plate is in addition to that attributable to random sampling.

5. The variance for samples is large also. This confirms findings reported previously (2, 3). The variance for samples is related to soil heterogeneity, to random sampling from the plot by two different randomizations, and to differences in the moisture content of the samples.

6. The mean squares for Error 1 are from one to six times those for samples. On the average, this error will not be less than the variance for samples because each plot estimate is based on two samples. It will be larger since it contains, in addition, that portion of the variance for between dates that is not explained by the correlations among numbers of bacteria and certain environmental changes.

7. The responses to moisture and time are enormous compared to any of the other effects listed. These have been considered fully in the previous paper.

Errors

Two errors are shown in the design of this experiment. Error 1 represents the unexplained portion of the variance for between dates. It is used to test plot or field effects. Obviously, the size of the error depends upon the precision of the plot estimate on each date. When the laboratory and field sampling procedures add a minimum to the error of replicate plates the variance for between dates may be largely assigned to correlations between numbers of bacteria and changes in environmental factors, such as moisture and time in

this experiment. If on the other hand the laboratory procedure is faulty, or if there is failure to compensate for heterogeneity of samples and random sampling by replications at each sampling step, Error 1 will be large and the sensitivity of the experiment lessened as a result. Further, the size of Error 1 depends upon the proportion of the variance that is assigned to definite relationships. In this experiment the variance for the correlation between bacteria and moisture is separated from Error 1. Accordingly, the error is reduced and the response to cropping is highly significant. Undoubtedly, the segregation of the variance for the correlation between bacteria and temperature from the error would provide a more sensitive test of the effect of cropping. This was not done in this experiment because soil temperature data were not available on all plots.

Error 2 represents the unexplained portion of the variance for within dates. It is used to test the significance of plot and laboratory sampling variances, and also to test Error 1. In this experiment it consists of the variance for all the interactions with plates except that for plates \times dilutions. Since it has been shown in Fig. 1 that the variances for certain sampling steps and interactions segregated in the analysis are only little larger than Error 2, the wisdom of splitting the degrees of freedom to this extent may be considered. The data for the complete experiment are summarized in Table II.

TABLE II

MEAN SQUARES FOR ERROR 2 AND PERCENTAGE INCREASES IN THE ERROR BY COMBINING CERTAIN DEGREES OF FREEDOM WITH ERROR 2

Column	1	2	3	4	5
Source of variance	Error 2	1 + plates + (dilutions \times plates)	2 + (dilutions \times subsamples)	3 + subsamples	3 + (dilutions \times plates)
Degrees of freedom	9	12	16	18	18
Fallow	7.4305	106.4	108.8	115.7	109.9
Wheat	9.8767	102.0	107.6	118.2	121.1
Barley	14.8226	100.6	108.6	117.2	107.5
Soybeans	7.7155	102.3	109.4	113.4	112.5
Sugar beets	8.7718	100.6	108.7	123.8	108.5
Corn	10.7492	99.1	110.2	117.4	121.0
Average in %	100	101.8	108.9	117.6	113.4

The mean squares for Error 2 for each crop are listed in Column 1. On the average these will be less than those for combinations of degrees of freedom that include Error 2. Combinations of Error 2 and different effects, expressed as percentages of Error 2, are shown in Columns 2 to 5. The bottom line gives averages for all crops. It is readily apparent that little is to be gained by splitting the 12 degrees of freedom for pairs of plates shown in the original design for the 24 plate unit. For practical purposes this error of replicate plates is the minimum laboratory error. The inclusion of other effects and

interactions in the error is largely a matter of judgment. Obviously, when the laboratory procedure is varied or a new medium is tested the error of replicate plates should be used in preliminary trials and the various interactions should be checked. Too, Chi square studies should be carried out to determine whether the variation within pairs of replicate plates conforms to expectancy on the basis of random sampling. Once it has been established that the population responds normally to the new variation in procedure the calculation may be reduced by segregating only the degrees of freedom for samples, subsamples, and dilutions, unless the remaining variance proves to be about as large as that for any of these effects. This would indicate failure to account for some disturbing factor.

The sensitivity of an experiment may be increased by reducing the error or by increasing the number of replications. In experiments involving plate count data the reduction of the error gives a more sensitive test of an effect than does increasing the degrees of freedom for error by replications. This may be illustrated by data from Plot 1, wheat.

Source of variance	Degrees of freedom	Variance	<i>F</i>	1% point	1% point with infinite degrees of freedom
Between dates	17				
r_{BM}	1	6136.7530	6.5475	8.53	6.64
Unexplained	16	937.2696			

The analysis shows that the variance for the correlation of bacteria and moisture is below the 1% level of significance and would still be even if the error represented an infinite number of degrees of freedom.

When time is included with bacteria and moisture in the correlation the unexplained portion of the sum of squares is reduced and the analysis becomes:

Source of variance	Degrees of freedom	Variance	<i>F</i>	1% point	1% point with infinite degrees of freedom
Between dates	17				
r_{BM}	1	6136.7520	11.6656	8.68	6.64
$R_{BMD} - r_{BM}$	1	7105.5072	13.5071	8.68	6.64
Unexplained	15	526.0560			

In this case the variance for the correlation of bacteria and moisture is highly significant. This increase in sensitivity is gained by including an additional independent variable which accounts for a large part of the variation unexplained by the simple bacteria-moisture correlation and consequently leaves a smaller variance for error.

The reduction of error is important when the factor studied is represented by a single degree of freedom and is likely to account for a smaller portion of the sum of squares than does moisture or time. Undoubtedly, this will be the case when the relation of root rot in cereals to numbers of bacteria in soil is studied. To be significant the variance must be 6.64 times that for the error with an infinite number of degrees of freedom.

In order to show the relative size of the sums of squares for certain groups of degrees of freedom used in the analysis the data for each crop are considered again. For simplicity of presentation, they are expressed on a percentage basis.

Crop	Total	Between dates	Moisture	Time	Error 1	Within dates	Repl. plates
Fallow	100	55.7	37.2	4.0	14.5	44.3	15.1
Wheat	100	63.5	25.2	16.6	21.7	36.5	10.1
Barley	100	65.6	16.2	25.8	23.6	34.4	10.9
Sugar beets	100	63.1	29.5	10.6	23.0	36.9	9.9
Soybeans	100	75.2	51.6	7.6	16.0	24.8	8.5
Corn	100	72.9	20.1	39.3	13.5	27.1	5.5

It may be noted that the sums of squares assigned to any heading differ in the different crops. This is most striking under between dates, where the sums of squares vary from 56 to 75%. The correlations among bacteria, moisture, and time account for from 40 to 60% of the total sums of squares in the various crops. Consequently, Error 1, or the unexplained portion of the sum of squares for between dates, is relatively small for each crop. Similarly the portions of the sums of squares for within dates not accounted for by samples, subsamples, dilutions, and their interactions are small. These unexplained portions are listed under replicate plates.

The following calculations based on the data from this study are presented to illustrate the relationship of the errors considered. The sums of squares, expressed as percentages and shown above, are averaged for all crops. Degrees of freedom are listed to represent 20 replications of the 24 plate unit from one plot. From these data relative variances are calculated for each heading. These follow.

	Total	Between dates	Moisture	Time	Error 1	Within dates	Repl. plates
Average	100	66.0	30.0	17.3	18.7	34.0	10.0
Degrees of freedom	479	19	1	1	17	460	240
Relative variances		3.47	30.0	17.3	1.1	0.07	0.04

The first logical step is to test the sums of squares for between dates against those for within dates.

Source	Sums of squares	Degrees of freedom	Variances	F	1% point
Between dates	66%	19	3.47	49.57	1.98
Within dates	34%	460	0.07		

Such a highly significant variance between dates may make that for within dates appear unimportant and lead to lack of proper care in plot and laboratory sampling procedures. However, the variance for within dates assumes new importance when the following data are considered.

Source of variance	Degrees of freedom	Variance	F	1% point
Between dates				
Moisture	1	30.0	27.27	8.40
Time	1	17.3	15.73	8.40
Error 1	17	1.1		
Within dates				
Samples, subsamples, dilutions, and interactions	220	.1091	2.616	1.38
Replicate plates	240	.0417		

1. The variance for replicate plates is the error for testing other laboratory and plot sampling procedures.

2. The average variance for samples, subsamples, dilutions, and their interactions is significantly larger than the variance for replicate plates. Therefore these sampling steps increase the error of any plot estimate.

3. On the average Error 1 will not be less than the largest variance for any step in the laboratory procedure.

4. The variances for the correlations among bacteria, moisture, and time are so large that any slight reduction in the sums of squares for within dates would cause little change in the significance of these responses. Obviously, any plot experiment designed to study changes in numbers of bacteria should provide information on these correlations. Whether this information is used, or the variance is merely removed from the error, will depend upon the object of the experiment.

Undoubtedly, the correlation between bacteria and the incidence of root rot in a plot would be less than that between bacteria and moisture or time. Consequently, the method must be sensitive enough to detect much smaller responses to environmental factors than those encountered in this study. This sensitivity will result from minimizing the error available for testing the response by giving careful consideration to plot and laboratory sampling procedures, as well as to bacterial responses to changes in environmental factors.

Acknowledgments

The authors are pleased to express appreciation to Dr. C. H. Goulden, Senior Agricultural Scientist, Dominion Rust Research Laboratory, Winnipeg, Man., and Honorary Lecturer in Statistics, The University of Manitoba, for critical advice in the statistical part of this study; to Mr. Robert Mitchell, The University of Manitoba, for preparing the charts; and to Mr. A. M. Brown, Assistant Plant Pathologist, Dominion Rust Research Laboratory, for photographing the illustrations.

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STUDIES OF CROWN ROT OF APPLE TREES¹

By MAURICE F. WELSH²

Abstract

The form of apple tree crown rot that occurs in the irrigated orchards of British Columbia is confined to the below-ground bark tissues of the tree. It has been encountered in trees of all ages and of all the commercial varieties.

Proof is given that this crown rot is caused by the fungus *Phytophthora cactorum* (L. & C.) Schroet. Typical symptoms of the disease have been reproduced in over 200 trees of various ages as a result of their inoculation with this fungus. Isolation has been possible only from the margins of active lesions, and has proved difficult even from these tissues. There is evidence that the activity of *P. cactorum* is inhibited in rotted tissues by the antagonistic effect of one or more secondary organisms.

The influence of soil moisture and temperature on disease incidence has been studied by field observations and by the inoculation of two-year-old trees under controlled conditions in Wisconsin tank equipment in the greenhouse. The effects of these two factors seem to be interrelated, with the highest incidence of disease in an almost saturated soil at the highest temperature imposed, 32° C. The influence of soil moisture is exerted particularly in the subsoil, rather than in the locus of crown rot attack.

Certain varieties of apple have been found to vary in their resistance to crown rot. Deep wounds have proved necessary to allow entry of the fungus into bark tissues.

* The additional information now available is being utilized in a search for improved means of combating the disease.

Introduction

Crown rot is a disease of considerable economic importance in the apple orchards of the Okanagan Valley in British Columbia and closely resembles certain of the crown and collar rot conditions of apple trees which have been reported from other regions. The present investigation has been designed to ascertain the nature of the disease and its specific cause, to elucidate the manner of operation of factors determining its incidence, and to seek improvements in the measures at present in use for its prevention and control.

DESCRIPTION OF THE DISEASE

Nature of the Injury to the Crown

The form of apple tree crown rot that is regarded as typical in the Okanagan Valley involves only the bark tissues of the tree below ground level. The attack is confined usually to those portions of the trunk and roots that lie within six inches of the surface of the soil. Only rarely are tissues at a greater depth, or above-ground tissues, attacked. The ability of the rot to spread

¹ Manuscript received June 23, 1942.

Joint contribution from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, (Contribution No. 710), and the Department of Botany, University of Toronto, Toronto, Ont. Based on a thesis presented in June, 1942, to the University of Toronto in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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through the tissues varies greatly in individual trees. When left undisturbed it may successfully girdle a large tree and spread several feet along the surface roots in the course of a few weeks. At the other extreme the spread may cease entirely after a small patch of rot has been produced, or a single root girdled. Not infrequently a tree may lose one or two small roots each year but never suffers extensive damage.

Crown rot usually can be detected only by careful examination. The rot is not apparent unless the tough outer layer of bark is scraped off. The death of the bark is evidenced by a browning and softening of all the remaining layers to the depth of the cambium. Recently rotted tissues are light yellow-brown in colour and are water-soaked regardless of the moisture level of the surrounding soil. As the rot becomes older the tissues turn a darker brown and may become slimy or may dry out, in accordance with the surrounding conditions. Actively spreading rot, as might be expected, has no definite margin but rather a transition from the dark brown colour of the older rot through successively lighter shades into the normal green of healthy bark. When the spread ceases a definite margin forms between the dark brown rotted tissue and the healthy tissue. Cracks often appear around this outer margin.

The sapwood beneath areas of rotted bark becomes discoloured, but there is no evidence of rotting of tissues beneath the cambium.

Infrequently and atypically the rot may extend as much as six inches above ground level. In such instances there is a zonation effect produced in the bark by the alternation of irregular layers of very light brown and darker brown tissue. These tissues have a firm, soapy consistency. At or near ground level there occurs a transition into the typical bright yellow-brown, water-soaked crown rot tissue.

Fig. 1 pictures the crown of a 15-yr. old Winesap tree which has been girdled by crown rot. In this tree the bulk of the crown rot lesion lies below ground, but it has extended a short distance above and displays the zonation type of rotting near its upper limit. *P. cactorum* was isolated from the margin of a light brown lesion in this tree.

Above-ground Symptoms

Since the bulk of the rotting occurs below the surface of the soil, the first noticeable indication of the disease is usually the appearance of secondary symptoms in the upper part of the tree, after the rot has progressed for some time.

The sequence of the appearance of secondary symptoms varies to some extent with the time of year at which the crown injury is sustained. The disease most frequently appears to be active in the early part of the summer. Then the first symptom is a bronzing, followed by a yellowing of the leaves already formed when the injury occurs. New leaves formed after this time usually fail to reach normal size. The bark develops a reddish tinge and the fruits may be small and conspicuously coloured. In the following season the



FIG. 1. Crown rot injury to the trunk of 15-yr. old Winesap tree. The outer bark layer has been removed to reveal extent of lesion. Tissues near root bases are typically rotted. The atypical zonation type of rotting has appeared above. FIG. 2. The above-ground symptoms of crown rot, including light-coloured, sparse foliage, reduction of terminal growth and premature colouring of fruit. Photograph by McLarty, 1923.

PLATE II



FIG. 3. Winesap tree two months after inoculation with *P. cactorum*. The surface bark has been removed to reveal the extent of the lesion. FIG. 4. A check tree in the same experiment, showing healing of the wound after two months.

yellowing and sparseness of foliage becomes more noticeable. It is accompanied by reduction of terminal growth. When the crown injury occurs later in the growing season no above-ground symptoms may appear until the following spring when the yellowing and sparseness of foliage become evident accompanied in the same season by the other described symptoms. The tree pictured in Fig. 2 exhibits the symptoms that usually appear during the season following crown injury.

Above-ground symptoms are produced only in those portions of the tree above the rotted sector of the crown. For this reason they are usually manifested first in a single limb of the tree. If complete girdling occurs, death of the entire tree will ultimately result.

When crown rot extends up to or above ground level, its presence can often be detected by the appearance of patches of liquid exudate on the bark. These may be immediately above or just within the upper margin of the rotted bark. This phenomenon has been described and pictured by McLarty (16).

OCURRENCE OF CROWN ROT IN BRITISH COLUMBIA

The Okanagan Valley forms the centre of the apple-growing industry in British Columbia. The greater part of the valley is entirely dependent on irrigation for maintenance of the orchards.

It is in this irrigated area, extending from the Vernon district south to the border of Washington, that crown rot most commonly occurs. The prevalence of the disease varies from district to district, and fluctuates in a given district from year to year. It is safe to say that a majority of the orchards are losing odd trees each year by crown rot attack. Periodically a more serious outbreak will occur in an individual orchard, involving within several years as much as half the planting. Trees of all ages are attacked with little preference although it is common belief that the first cropping of a young tree renders it particularly susceptible. The prevalence of the disease and the fact that it injures or kills trees whose replacement or recovery requires a number of years, have conspired to make this one of the most serious plant disease problems in the region.

Several other types of crown and trunk injury are known in the Okanagan Valley. Winter injury of apple occurs periodically in the northern sections of the valley and usually can be distinguished readily from crown rot on the bases of both occurrence and symptoms. Mechanical injury, produced by disking or by rodents, is also readily recognizable. An additional condition frequently encountered in both young and old trees is a superficial browning of the bark of the crown below ground level. The depth of this tissue killing varies greatly. Infrequently it is found penetrating to the depth of the cambium over a limited area. Although this bark degeneration resembles that found in typical crown rot, there appears to be no correlation between the occurrences of the two disorders. The cause of this surface browning remains obscure.

PREVIOUS INVESTIGATIONS OF CROWN ROT IN BRITISH COLUMBIA

Crown rot has been the subject of observation and investigation by plant pathologists in the Okanagan Valley for about 20 yr. Many of the data accumulated have been included in reports by McLarty (15, 16) and Roger (20, 21). The information gained in their researches included the following.

Both in its symptom picture and in the circumstances of its occurrence crown rot could be distinguished readily from low temperature injury. Its incidence showed no correlation with the use of arsenical sprays and the symptoms differed from those reported as arsenical injury symptoms by Swingle and Morris (24) and by Hotson (12).

Most or all of the initiation of crown rot occurred during the summer months. The lesions enlarged gradually, suggesting fungal or bacterial action. However all attempts to isolate a causal organism were unsuccessful. No isolate appeared with a significant degree of consistency, and inoculation experiments with the more common isolates in no case produced crown rot symptoms.

Although the distribution of the disease in certain orchards indicated varying resistance among the interplanted varieties, the disease could be found from time to time in all the commercial varieties.

With few exceptions the outbreaks of crown rot could be correlated with the existence of excess moisture conditions in the orchard soil. Reduction of soil moisture, by adjustment of irrigation application or by drainage, consistently reduced the severity of the outbreak. The precise effect of this excess moisture was not determined. Furthermore orchards were encountered in which soil moisture conditions appeared to be ideal for crown rot occurrence but in which the trees remained disease-free. When an outbreak of disease did occur in an orchard the selection of trees for attack often was unpredictable. When crown rot was once initiated the extent of the lesions varied greatly from tree to tree, in certain trees remaining as small isolated patches of rot, in others spreading over the entire surface of the crown and surface roots. It appeared, therefore, that additional factors were operative in determining disease incidence. The nature of these additional factors remained obscure.

Experimental Work

ISOLATION AND PROOF OF PATHOGENICITY OF *Phytophthora cactorum*

SCOPE OF THE ISOLATION PROGRAM

In the course of four summers' isolation work, diseased material has been collected from a total of 96 trees in orchards scattered through the various apple-growing districts of the Okanagan Valley, from Osoyoos on the border of the State of Washington to the Vernon district approximately 100 miles to the north. In the first two seasons all isolates were retained for possible further study. However in view of recent demonstrations of the importance of species of *Pythium* and *Phytophthora* in the etiology of root rot diseases, particular attention was paid to the isolation of species of these two phyco-

mycete genera and to tests of their pathogenicity. In its later phases the investigation was limited to the isolation and study of the single species, *Phytophthora cactorum* (L. & C.) Schroet.

ISOLATION TECHNIQUES EMPLOYED

A standard technique has been evolved for isolation of the fungus from crown rot tissue. Blocks of bark tissue are removed from the crowns of diseased trees, each block being so cut that it includes the border of diseased and healthy tissue in the region where the transition appears to be most gradual. The blocks are cut to include all layers of bark to the depth of the cambium. This material is brought into the laboratory as quickly as possible. In the laboratory the block is split to make possible the removal of small pieces of previously unexposed tissue from the border of the lesion. These pieces of tissue are planted on potato dextrose agar.

The apple fruit isolation method described by Tucker (27) has been utilized in some later experiments. It is somewhat more laborious however and does not appear to allow more consistent isolation of *P. cactorum* than does the method outlined above.

In the earlier stages of the isolation program variations of the standard method were tested. Plantings were made of rotted tissue from well behind the margin of obvious infection, and from the diseased tissues of trees in which the spread of rot obviously had been halted. Several types of culture media were employed. These variations were discarded when no advantage appeared to accrue from their use.

RESULTS OF ISOLATION

In the earlier stages of the investigation when isolation was being made from all types of tissue, a variety of organisms was recovered from crown rot tissues. Included were species of *Fusarium*, *Helminthosporium*, *Alternaria*, other unidentified hyphomycetes, and a number of fungi that were not induced to fruit in culture. Bacterial colonies also appeared frequently. Three phycomycetes were isolated. The first isolate obtained of each was used in pathogenicity tests. In the writer's stock culture catalogue these were designated as Isolates 3a, 229a, and 339c. Isolate 3a was obtained from two trees in well separated orchards. It is characterized by coenocytic mycelium and bulbous chlamydospores. Reproductive structures have never been observed. Isolate 339c is a species of *Pythium*. Isolate 229a has been identified as *Phytophthora cactorum* (L. & C.) Schroet. This identification was made for the writer by Dr. T. C. Vanterpool, University of Saskatchewan, Saskatoon, Sask., in October, 1939, by comparison with a type culture of *P. cactorum* from the collection of S. F. Ashby at Kew.

Since the discovery of crown rot usually awaits the appearance of secondary above-ground symptoms in the attacked tree, opportunities to isolate from active lesions have been infrequent. When it has been possible to make isolations from the margins of such freshly rotted bark, only two organisms have been isolated, one bacterium and the other the phycomycete *Phytophthora*

cactorum. The bacterium is represented in the stock culture catalogue by Isolate 452b. It can be isolated occasionally from these marginal tissues, but is yielded much more frequently from the darker brown rotted tissues immediately behind the border of the lesion. On the other hand isolation of *P. cactorum* has been successful only when plantings have been made of light brown tissues at the extreme margin of the active lesion.

P. cactorum has been isolated from natural occurrences of crown rot in seven trees, in six orchards scattered through the length of the Okanagan Valley, in the Vernon, Kelowna, Summerland, and Osoyoos districts, and on the Dominion Experimental Station near Summerland.

Crown rot lesions produced by the inoculation of healthy trees with *P. cactorum*, whether in the greenhouse or out-of-doors, have yielded the causal fungus more consistently than have the natural occurrences of the disease. This greater measure of success undoubtedly has been attained because it is possible to keep inoculated trees under observation, and thus select tissue for isolation while the rot is spreading rapidly.

INOCULATION EXPERIMENTS, 1938, 1939

Inoculation experiments were begun in 1938 and continued throughout the summer of 1939, using essentially the same technique and test material in the two seasons. These experiments were designed to test the pathogenicity of various isolates that had been yielded from crown rot lesions, with particular attention paid to the isolated phycomycetes.

The technique of inoculation in all 1938 and 1939 experiments consisted of the lifting of flaps of bark and the insertion of inoculum beneath. The flaps were bound back in place with raffia. The inoculum consisted of mycelial wads removed from cultures grown in potato dextrose agar.

For most of these early experiments the inoculations were made into one- and two-year old apple trees growing in sand culture in the greenhouse, with automatic irrigation which allowed the sand about the crowns to remain constantly moist although never saturated. Various combinations of scion and rootstock were included. The scion varieties were McIntosh and Delicious. The rootstocks included several Malling stocks and seedling stocks of Canada Baldwin and French Crab. In each experiment care was taken so to distribute the inoculations that the various stock-scion combinations were divided equally among the included tests. The inoculation in each case was made at the junction of scion and rootstock, situated just beneath the surface of the sand. These trees were used for tests of the pathogenicity of the two phycomycetous isolates designated 3a and 229a*, the most frequently isolated species of *Fusarium*, and two unidentified non-sporulating isolates.

* Isolate 229a will be known throughout the remainder of this paper as *P. cactorum* isolate No. 1, although the fungus remained unidentified when the 1938 and 1939 experiments were performed.

Additional tests were made using older trees growing out-of-doors. Two 20-yr. old trees were inoculated by essentially the same technique used for inoculation of the young trees, one tree with Isolate 3a, the second with *P. cactorum* isolate No. 1. Peat was packed about the crowns of the trees, and kept moist by means of a constantly dripping water supply. The crowns of two trees were wounded and packed with moist peat in the same manner to serve as checks.

In addition an effort was made to test the pathogenicity of the two phycomycetous isolates by above-ground inoculations of a bearing Rome Beauty tree. Flaps of bark were lifted on the main limbs of the tree, and inoculum was inserted beneath. The wounds were bathed with wicks of cheese-cloth fed from suspended vessels of water above.

RESULTS OF 1938 AND 1939 INOCULATIONS

The results of the inoculation of young trees in sand culture indicated an ability of Isolates 3a and *P. cactorum* No. 1 to cause rotting of apple bark tissues. Wherever these organisms were inserted into wounds at least the lifted bark was rotted. Usually there occurred a limited spread of rot into surrounding bark tissues. The inoculation with the *P. cactorum* isolate produced as a rule a more extensive rotting. In three trees, inoculation with this organism caused complete girdling of the crown. In check trees where sterile agar was substituted for fungal inoculum and in trees inoculated with the remaining three isolates, the lifted bark flaps remained sound and the wound callused rapidly.

The inoculation of limbs of the older Rome Beauty tree with the Isolates 3a and *P. cactorum* No. 1 resulted once again in a rotting of lifted bark and in a limited spread of the rot into surrounding bark tissues. Both organisms were reisolated from the border of this rotting tissue.

The first production of typical crown rot symptoms resulted from the inoculation of the crown of a 20-yr. old tree with the *P. cactorum* isolate. The inoculation was made on April 3, 1939. By October 15, 1939, the tree was two-thirds girdled by typical crown rot just beneath the surface of the peat. *P. cactorum* was reisolated from the margin of the spreading rot on October 15. The simultaneous inoculation of a neighbouring tree with Isolate 3a did not produce any crown rot. The two check trees, wounded but not exposed to inoculum, also remained unattacked.

INOCULATION EXPERIMENT, 1940

A block of 17 trees in the grounds of the Dominion Laboratory of Plant Pathology at Summerland was set aside for tests of the pathogenicity of *P. cactorum* isolate No. 1, Isolate 3a, and Isolate 339c. The trees, which were seven years old and in bearing, included the four varieties: Newtown (three trees), McIntosh (four trees), Delicious (four trees), and Winesap (six trees). All were growing on unknown rootstocks. Soil and water conditions appeared to be uniform throughout the block. No crown rot had been known to occur in this block in previous years.

The method of inoculation was essentially that employed in the 1938 and 1939 experiments. Two long vertical slits were cut in opposite sides of the crown of each tree and the inoculum inserted beneath the bark throughout the length of the slits. In check trees sterile agar was inserted in the vertical slits. The original soil was replaced about the wounded crowns. A condition of ample, but never excessive, soil moisture was maintained about these trees throughout the experiment. Two sets of inoculations were made.

On April 5 and 6, 1940, inoculations were made as follows:

Isolate 3a.....1 Newtown, 1 McIntosh, 2 Delicious, 2 Winesap.

Isolate 339c.....1 Newtown, 1 Winesap.

P. cactorum.....1 Newtown, 2 McIntosh, 1 Delicious, 3 Winesap.

No crown rot resulted from inoculations with Isolates 3a and 339c. There appeared only a restricted rotting of the bark lifted for insertion of inoculum. In the two uninoculated check trees even this lifted bark remained healthy (Fig. 4).

Typical crown rot was produced in the three Winesap trees inoculated with *P. cactorum*. Complete girdling of the three trees occurred within three months of inoculation, with a spread of the rot through the bark tissues of both scion and rootstock portions of the crown (Fig. 3).

On July 25 all trees in which crown rot had not been produced by the earlier inoculation were reinoculated with *P. cactorum*. These included three trees each of the four varieties. The two check trees were again wounded without application of inoculum. The three Winesap trees suffered complete girdling as a result of this inoculation. One of these trees was girdled within three weeks. Again no crown rot was produced in the varieties Newtown, McIntosh, and Delicious.

Thus the inoculation of the six Winesap trees with *P. cactorum* resulted in the initiation of typical crown rot in every case. Three of these were trees whose earlier inoculation with the Isolates 3a and 339c had been ineffective.

SUMMARY OF INOCULATION RESULTS, 1940-42

In 1940, 1941, and 1942 crown rot was produced in a total of 201 trees as a result of their inoculation with *P. cactorum*. The details of the various experiments are given in other sections of this paper. In the course of varietal resistance studies the disease was produced in 58 bearing trees in the field and 50 one- and two-year old trees in the field. The soil environment studies carried out under controlled conditions in the greenhouse resulted in the production of disease in 93 two-year old trees.

In all the experiments outlined *P. cactorum* has been reisolated from the margin of actively spreading rot in representative trees. The symptoms resulting from inoculations with *P. cactorum* are in every respect typical crown rot symptoms. The same tissues are attacked in the same locus and the progressive stages of tissue breakdown appear to be identical.

Microscopic Examination of Crown Rot Tissues

In an effort to demonstrate further the association of *P. cactorum* with crown rot, a microscopic examination has been made of samples of freshly rotted tissue in all collections of diseased material made in the summers of 1940 and 1941. The staining of tissue scrapings with cotton blue or acid fuchsin in lactophenol has revealed the constant presence of coenocytic mycelium growing intercellularly and intracellularly in freshly rotted tissues. This mycelium corresponds well with that of *P. cactorum* in culture. Frequently it has been found in rotted tissues when attempted isolation from the same tissues has indicated that they are sterile or harboured only the bacterium 452b.

SOIL MOISTURE AND TEMPERATURE AS FACTORS INFLUENCING DISEASE OCCURRENCE

During the course of these investigations of crown rot several lines of approach have been followed in the search for further data on the influence of soil moisture and soil temperature on crown rot incidence.

In particular, with the parasitic nature of the disease established and the causal organism identified, it has been possible to design experiments in which the progress of the disease could be followed under various controlled conditions of soil moisture and soil temperature.

Field inoculation experiments have provided some additional data on the influence of soil moisture and temperature. In addition, field observations of the natural occurrence of the disease have been continued.

TEMPERATURE AND MOISTURE RELATIONS OF *Phytophthora cactorum* IN PURE CULTURE

As a preliminary to studies of the effect of soil moisture and temperature on the pathogenicity of *P. cactorum*, tests have been made of the temperature-growth relations of the fungus and of its response to graded relative humidity levels while growing in pure culture.

Temperature Studies

P. cactorum isolate No. 1 was grown on plates of Leonian's malt extract, yeast extract agar (14) at the temperatures 2, 4, 6.5, 8, 14, 21, 25, 27, 30, 32, and 34° C. Inoculum disks of uniform size were planted at the centres of the plates. Colony diameters were measured daily for a period of eight days. The experiment was performed in triplicate.

The results are illustrated in Fig. 6. The minimum temperature for growth under the conditions imposed was shown to be between 4 and 6.5° C. The rate of growth increased steadily with rise in temperature as far as 27° C. The maximum temperature approximated 32° C., with death resulting from eight days' exposure to this temperature. Even at 30° C. growth became slower and somewhat irregular as the exposure continued.

Relative Humidity Studies

P. cactorum was grown in culture with the relative humidity of the enclosing chambers governed by the dehydrating activity of varying concentrations

of sulphuric acid. The culture medium used was 3% potato dextrose agar. The concentrations of sulphuric acid required for humidity regulation have been listed in a table by Stevens (23) and presented graphically by Hopp (11). Their figures, which were in good agreement, were accepted and used by the writer. The more dilute solutions of sulphuric acid were sterilized before use.

The glassware employed for each culture and humidity chamber consisted of the lower half of a 7 cm. Petri plate and a complete 10 cm. Petri plate, the former being placed within the larger chamber prior to the sterilization of the whole.

A 12 cc. portion of melted agar was poured into each 7 cm. enclosed plate. Immediately thereafter, 12 cc. of sulphuric acid of the required strength was poured into the outer chamber. Twenty-four hours later a small disk of *P. cactorum* mycelium was planted in the centre of the plate of agar.

The humidities sought were 0.5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% of saturation. Each test was performed in duplicate. One additional pair of plates contained no solution in the outer chamber.

In order to give more significance to the results with *P. cactorum*, an identical series was set up, using as test organism a species of *Helminthosporium* which causes a leaf blight of Russian thistle under dry summer conditions in the interior of British Columbia. The growth rate of these two fungi on potato dextrose agar was known to be approximately the same. The *Helminthosporium* was exposed to 6 of the 11 relative humidities, viz.: 0.5, 20, 40, 60, 80, and 100%.

(a) Growth of the Fungus

The rate of growth of *P. cactorum* at each humidity during a period of seven days is recorded in Table I. The growth ceased before the seventh day in colonies exposed to relative humidities below 90%. The growth rate of all the colonies was comparable, and remained steady until it was halted by complete desiccation of the substrate. The final diameter reading of the colonies was frequently a record of one sector only, the substrate beneath the remainder of the colony having already dried. The amount of aerial growth appeared to be the same at all relative humidities.

It is evident that no significant difference in growth rate was effected by variation of the relative humidity of the culture chamber as long as the agar substrate remained moist.

The *Helminthosporium* series exhibited the same uniformity of growth rate at all humidity levels.

(b) Survival of the Fungus

A daily transfer was made from each plate, beginning shortly before the complete desiccation of the medium and the accompanying cessation of fungus growth. Where the medium dried unevenly over the surface of the plate, transfers were made from both the moist and the dry areas. When the fungus could be transferred no longer, a final attempt was made to revive the colonies by flooding them with cooled melted agar.

TABLE I

THE GROWTH OF *P. cactorum* IN CULTURE UNDER VARYING CONDITIONS OF ATMOSPHERIC HUMIDITY. DAILY READINGS OF COLONY DIAMETER IN MILLIMETRES. FIRST READING TWO DAYS AFTER INOCULATION

Relative humidity, %	Colony diameter, mm.							
	7	12	23					
0.5	7 7	14 14	22 22					
10	7 6	14 14	17 20					
20	8 7	15 15	22 22	25				
30	6 7	14 13	20 20	27				
40	7 7	15 15	22 25	24 30				
50	7* 8*	15 16	19 20					
60	8** 7	16 13	24 21	32 29	35			
70	7** 8**	16 17	24 27	32 35	38 37			
80	7 8	15 16	23 23	30 30	38 38	46 47	55 53	
90	7 9**	14 16	20 23	26 31	(Contaminated)		(Dry after 3 more days)	
100	8 6	15 12	21 19	30 27	35 34	40 40	49 47	(Growth continued) (Growth continued)
Check	9 6	15 18	22 24	28 31	35 38	43 45	51 54	(Growth continued) (Growth continued)

* First inoculation unsuccessful. Plate reinoculated one day later.

** First inoculation unsuccessful. Plate reinoculated two days later.

P. cactorum in all cases remained alive as long as the substrate immediately beneath it remained moist. At all relative humidity levels below 60% the drying of the substrate resulted not only in cessation of fungus growth but also in its immediate death. This was strikingly evident where only a portion of a single plate was dried. Transfers then were unsuccessful from the dry area, although successful from the remainder of the colony. One of the two colonies at 60% humidity was revived two days after growth had ceased. Both colonies at 70% humidity were revived after two days. Of the colonies at 80% humidity, one remained viable for two days and the other for four days. One of the colonies at 90% humidity was lost by contamination. The other was revived five days after cessation of growth.

Microscopic examination of mycelial wefts from the *P. cactorum* colonies revealed that reproductive structures had not been produced in most of the colonies before the medium became dry, so that the survival of the colonies was a function of the mycelium alone. The single exception was a colony exposed to 40% humidity, which produced small numbers of oospores. These oospores apparently did not remain viable after the desiccation of the medium beneath. At the four lowest humidities the mycelium suffered almost complete disintegration in the three days following final drying of the substrate.

The colonies of *Helminthosporium* displayed a much greater resistance to desiccation. Of the colonies at 0.5% humidity one was successfully revived three days after, and the other, four days after desiccation of the substrate. A colony at 20% humidity yielded a successful transfer after 14 days. At higher humidities the colonies remained viable three weeks after the desiccation of the substrate. This fungus began to sporulate four days after the plates were inoculated, so that the colonies grown at 0.5 and 20% humidity were unable to sporulate before the desiccation occurred. Thus the survival of these colonies must have been a function of the mycelium alone.

SOIL MOISTURE AND TEMPERATURE STUDIES UNDER CONTROLLED CONDITIONS

In an effort to determine precisely the soil conditions favouring crown rot incidence, young apple trees have been exposed to inoculum of *P. cactorum* while growing under various experiences of soil moisture and soil temperature.

Experiment I

Wisconsin tank equipment was utilized for control of soil moisture and soil temperature levels. Seventeen combinations of moisture and temperature were imposed. Tank equipment was available for the maintenance at a given time of six soil temperature levels, with eight trees accommodated at each temperature. Thus 48 trees were included at each temperature series. The temperature levels were adjusted to give the best possible series between the previously determined minimum and maximum temperatures for growth of *P. cactorum* in culture. In order to combine tests of soil moisture with the soil temperature studies, three successive series were set up, at three varying soil moisture levels. Thus eight trees were subjected to each of the 17 soil moisture-soil temperature experiences.

Sterilized soil was used throughout. The soil moisture level was adjusted by the thorough mixing of measured amounts of water with the soil in the cans before the trees were planted. The moisture levels were maintained during the course of the experiment by periodic weighings of the cans and the addition of water to make up the original weight. Where the soil was being maintained at a moisture level too low to make this feasible, an alternative criterion of moisture level was provided by the readiness with which the soil at the various moisture levels would mould into a ball, and the readiness with which this ball would crumble thereafter.

Infestation of the soil was achieved by the introduction of corn-meal, sand, canned pea medium on which the fungus had been cultured for three weeks.

This material was added at the rate of 5% by weight to the soil which occupied the upper one-quarter of each can, in contact with those tissues that usually form the locus of crown rot attack.

The set of Wisconsin tanks was contained in a single greenhouse. The temperature of the house was maintained as constantly as possible at 65° F., midway between the extremes of soil temperature included in the experiment. The atmospheric humidity was kept as low as possible in an attempt to approximate Okanagan Valley summer conditions. It varied between the limits 35 and 50% of saturation while the experiments were in progress. To avoid excessive evaporation and loss of heat from the surface of the soil, a thick wad of non-absorbent dressing cotton was spread over the entire soil surface in each can and packed about the trunk of the tree.

The test trees were two years of age, growing on French Crab rootstocks. In each individual test equal numbers were used of the two varieties Canada Baldwin and Grimes Golden. The trees were allowed to remain out-of-doors for a necessary hardening-off period during the early part of the winter. Four weeks before the setting-up of each series, the required trees were brought into a warm humid greenhouse. The roots were washed thoroughly, the roots and branches pruned, and the trees potted in sterilized soil. The emergence of the trees from dormancy was not uniform. The four-week period allowed complete unfolding and expansion of the leaves in certain trees, while others remained dormant. In general the Canada Baldwin trees responded more rapidly than the Grimes Golden. Considerable care was taken to apportion the dormant and vigorous trees equally to the various individual tests.

When the trees were repotted for subjection to the conditions of test, care was taken to plant each tree at such a depth that at least two inches of the scion portion of the crown lay beneath the surface of the soil. A narrow vertical slit was cut in the bark of the crown of each tree, to expose cambial tissue from ground level to the depth of the stock-scion junction.

Each series was continued for a period of 24 days. The crowns of the trees were uncovered and examined three days after insertion of the wounds, and at two-day intervals thereafter.

In the first series a soil moisture level approaching saturation was attained, by the addition of approximately 2 l. of water to 16 lb. of dry soil. A soil moisture determination made at the end of the experiment by the method of Riker and Riker (19) revealed the percentage of saturation to be approximately 96.

In the second series a moisture level was sought that would be slightly below the optimum level for the welfare of the host plant. Approximately 700 cc. of water was mixed with 16 lb. of dry soil. The moisture level, determined at the end of the experiment, proved to be approximately 61% of saturation.

In the third series a moisture level close to the optimum for the welfare of the host plant was sought. Approximately 1 l. of water was mixed with

16 lb. of dry soil. The moisture level so attained was approximately 71% of saturation.

Results

A disease rating was evolved, based on the degree of below-ground girdling suffered by the individual trees. In the compilation of the tables, each unit represents the one-quarter girdling of a single tree. The maximum figure attainable in any single test is 16, representing the complete girdling of the four included trees of the one variety. The results of the entire 24-day experiment are detailed in Tables II and III. In order to give some idea of the speed of the disease attack, the results at the end of 15 days have been included.

Examination of Tables II and III reveals the following important data.

1. Within the temperature range 4 to 23° C., manipulation of the soil moisture appeared to exert little influence on the amount of crown rot produced.

2. At all three moisture levels there was an increase in amount and rapidity of crown rot attack with rise in temperature from 4 to 23° C.

3. In the soil approaching saturation, further increase of temperature from 23 to 32° C. resulted in still greater rapidity and extent of crown rot attack. The combination of this high soil moisture level with a soil temperature of 32° C. allowed the girdling of all trees in the test more rapidly than any other combination of conditions imposed.

4. At the two lower soil moisture levels, further increases of soil temperature above 23° C. did not result in increased incidence of the disease. Indeed in soil at 61% saturation, such an increase of soil temperature resulted in a reduction of the disease, to the limit of complete disease escape at 32° C.

P. cactorum was recovered from the margin of the lesions in one or more infected trees from each of the soil moisture-soil temperature experiences in which the disease had been produced.

Experiment II

An attempt was made to separate the effects of soil moisture level on host predisposition to crown rot and on host-parasite relationships in the locus of infection.

The Wisconsin tanks were again used to provide controlled soil moisture and temperature levels. The trees used were two years of age, Canada Baldwin and French Crab rootstocks. The conditions of experiment varied from those of the last experiment chiefly in the fact that in this experiment the moisture supply to the roots of the trees and the moisture level of the surface soil about the crowns of the trees were manipulated independently. Four soil moisture experiences were included with 10 trees exposed to each:

- (1) The soil throughout the can at a high moisture level (approximately 85% of saturation).

(2) The lower soil about the roots at a high moisture level (85%), the soil in the surface one-quarter of the can at a low moisture level (35%).

TABLE II

THE INFLUENCE OF SOIL MOISTURE AND SOIL TEMPERATURE ON THE PRODUCTION OF CROWN ROT BY *P. cactorum* IN CANADA BALDWIN TREES UNDER CONTROLLED CONDITIONS

Temperature, °C. and °F.	Soil moisture		
	High Series 1 (96%)	Intermediate Series 3 (71%)	Low Series 2 (61%)
Results 15 days after wounding of the crowns			
32° C. (90° F.)	4,4,4,4.....16*	2,0,0,0.....2	0,0,0,0.....0
29 (84)	4,4,4,2.....14	3,2,0,0.....5	4,2,0,0.....6
23 (73)	4,4,2,1.....11	4,4,2,0.....10	2,2,0,0.....4
17 (63)	4,0,0,0.....4	2,2,1,0.....5	0,0,0,0.....0
11 (52)	0,0,0,0.....0	2,0,0,0.....2	0,0,0,0.....0
4 (39)	0,0,0,0.....0		0,0,0,0.....0
Results 24 days after wounding of the crowns			
32° C.	4,4,4,4.....16*	4,4,2,0.....10	0,0,0,0.....0
29	4,4,4,4.....16	4,4,1,0.....9	4,4,1,0.....9
23	4,4,4,1.....13	4,4,4,4.....16	4,4,2,1.....11
17	4,1,0,0.....5	4,4,4,0.....12	4,0,0,0.....4
11	1,0,0,0.....1	4,1,0,0.....5	4,0,0,0.....4
4	0,0,0,0.....0		0,0,0,0.....0

* Arbitrary disease rating: each unit = one-quarter girdling of a single tree. Four trees included in each test.

TABLE III

THE INFLUENCE OF SOIL MOISTURE AND SOIL TEMPERATURE ON THE PRODUCTION OF CROWN ROT BY *P. cactorum* IN GRIMES GOLDEN TREES UNDER CONTROLLED CONDITIONS

Temperature, °C.	Soil moisture		
	High Series 1 (96%)	Intermediate Series 3 (71%)	Low Series 2 (61%)
Results 15 days after wounding of the crowns			
32	3,1,0,0.....4*	3,1,0,0.....4	0,0,0,0.....0
29	4,0,0,0.....4	0,0,0,0.....0	0,0,0,0.....0
23	4,2,0,0.....6	0,0,0,0.....0	0,0,0,0.....0
17	0,0,0,0.....0	0,0,0,0.....0	0,0,0,0.....0
11	0,0,0,0.....0	0,0,0,0.....0	0,0,0,0.....0
4	0,0,0,0.....0	0,0,0,0.....0	0,0,0,0.....0
Results 24 days after wounding of the crowns			
32	4,4,4,4.....16*	4,4,0,0.....8	0,0,0,0.....0
29	4,4,2,0.....10	4,0,0,0.....4	2,0,0,0.....2
23	4,4,3,1.....12	4,3,0,0.....7	0,0,0,0.....0
17	0,0,0,0.....0	1,0,0,0.....1	1,0,0,0.....1
11	0,0,0,0.....0	0,0,0,0.....0	2,0,0,0.....2
4	0,0,0,0.....0		0,0,0,0.....0

* Arbitrary disease rating: each unit = one-quarter girdling of a single tree. Four trees included in each test.

(3) The soil throughout the can at a low moisture level (45%).

(4) The lower soil at a low moisture level (45%), the surface soil at a high moisture level (85%).

Where it was necessary to maintain the two differing moisture levels in a single can, a layer of coarse gravel served as an effective insulation between.

The pretreatment of the trees again involved their exposure to a cold spell out-of-doors, their removal indoors, and their growth for one month in a warm humid greenhouse. The trees destined for exposure to a high soil moisture supply (Groups 1 and 2 above) were grown during this preparatory period at approximately the same high moisture level (85 to 90%). The trees destined for exposure to a low moisture supply (Groups 3 and 4) were grown during the preparatory period in a soil whose moisture level was about 50% of saturation.

At both pretreatment soil moisture levels all the trees were displaying green buds two weeks after introduction to the greenhouse, and were in full leaf at the time of transference to the Wisconsin tank equipment. At the time of transference, however, there was a striking difference in the degree of vigour of the trees that had been subjected to the two pretreatment moisture levels. At the lower moisture level (50%) both the root systems and the foliage of all trees were healthy. These trees wilted for a short time after transplanting but recovered and remained quite vigorous during the period of the experiment. At the higher soil moisture level on the other hand, most of the newly developed succulent roots of the trees were killed during the preparatory period. The leaves of many of these trees were stunted and scorched. In all the trees used, however, the bark of the crown and trunk remained green and succulent.

In all other respects the methods used and the conditions of the experiment were exactly those outlined in Experiment I. The inoculum again consisted of a corn-meal, sand, canned pea mixture on which *P. cactorum* had been grown for a period of three weeks. This was mixed with the soil in the surface one-quarter of each can at the rate of 3% by weight. A soil temperature of 27° C. was maintained in all cans throughout the experiment.

Results

Observations were made of the crowns of all trees at intervals of several days for a period of four weeks. The incidence of disease at the end of this time was as follows.

Group 1 (high/high)—8 trees girdled, 1 tree $\frac{1}{2}$ -girdled.

Group 2 (low/high)—10 trees girdled.

Group 3 (high/low)—1 tree girdled, 2 trees $\frac{1}{2}$ -girdled.

Group 4 (low/low)—No disease.

The bulk of the crown rot occurred within 10 days of the wounding of the crowns. Observation at the end of this period revealed complete or partial girdling of nine trees in the first group, eight trees in the second, and none in the other two groups.

Isolation was attempted, and *P. cactorum* was isolated from two of the diseased trees in each of Groups 1 and 2.

Experiment III

In this experiment vigorous trees, previously grown at the low soil moisture level (45% of saturation), were exposed to the condition of high soil moisture supply and low surface soil moisture. It was hoped that thereby it might be determined whether the predisposition of trees in Experiment II was the direct result of the high moisture supply, or a function rather of the low vigour of the trees grown under such conditions.

Twelve trees were inoculated in the test. All were in full leaf and vigorous at the time of transference to the Wisconsin tank equipment. Eight trees were subjected to a high soil moisture supply (90% of saturation). The four remaining trees served as checks, growing in soil whose moisture level approximated 45% of saturation. The surface soil surrounding the crowns of the 12 trees was maintained at the low level of 35% of saturation. A soil temperature of 27° C. was maintained. All trees were exposed to fungal inoculum in the surface soil and wounded by the usual method. The experiment was continued for 24 days.

Results

At the end of 12 days one of the eight trees exposed to high subsoil moisture was girdled, and a second partially girdled. Leaf scorch symptoms had appeared in these eight trees. The trees exposed to low subsoil moisture showed no leaf scorch and were unattacked by crown rot. However, by the end of 24 days, crown rot had appeared in only one more tree exposed to high subsoil moisture, while three of the four trees exposed to low subsoil moisture had been attacked.

DATA FROM FIELD OBSERVATIONS AND FROM FIELD INOCULATION STUDIES

The writer's observations of the natural occurrence of crown rot have been closely in agreement with those made by previous workers. The most serious outbreaks as a rule have appeared in orchards receiving seepage water from higher land. Usually this high moisture level is manifest both in the surface soil about the crowns of the trees and in the subsoil from which the tree is receiving the bulk of its moisture supply.

Apparent exception to this general correlation has been encountered in certain orchards. In one of these orchards observations were made at intervals for a year. The observations revealed that although the orchard lay on a side-hill, and although the surface soil was very dry during a large part of the growing season, seepage conditions maintained a high subsoil moisture level. The crown rot occurrence appeared to coincide with the seepage area.

In one of the varietal resistance experiments carried out in 1941 there arose an opportunity to study the relation of low surface soil moisture to crown rot spread. Crown rot had been initiated in a number of trees by inoculation with *P. cactorum* at a time when the soil was very moist. By the third week

of July a period of hot dry weather had reduced greatly the surface soil moisture level while the subsoil moisture level remained high. The spread of crown rot continued. Samples of soil were removed from the immediate vicinity of the lesion margins and their moisture levels were tested by the method of Riker and Riker (19). During a four day period while the moisture content of the soil about a single root lesion dropped from 32.0 to 11.3% of saturation, the lesion margin was extended by three-quarters of an inch throughout its length. The spread of this lesion continued for several weeks after the second observation. The same two observations were made in a second tree in a drier portion of the same orchard. Here the lesion margin was extended by one-half inch while the soil moisture level dropped from 23.8 to 10.8% of the moisture-holding capacity. The spread of this lesion ceased several days after the second observation.

Data from both field observations and field inoculation experiments have indicated that crown rot is most active during periods of hot weather. The successful isolation of *P. cactorum* from naturally-occurring crown rot has been confined to the months of July and August, with the single exception of an isolation made on May 31, 1940, in the Osoyoos district at the southern limit of the Okanagan Valley in Canada.

In the course of a varietal resistance experiment inoculations were made of 40 random-selected trees on December 5, 6, and 7, 1940. No crown rot had resulted from this set of inoculations by July 1, 1941. Repeated inoculations of these trees on July 4 and August 8 resulted in the appearance of crown rot in 23 of the 40 trees. An inoculation of 41 other trees in this same block on April 22 and 23 yielded 10 cases of crown rot. Two later inoculations of the remaining 31 trees in early July and in early August produced crown rot in an additional 16. Thus the December inoculation failed to produce crown rot in a number of trees which were proved to be susceptible by the later inoculations.

The latter part of August and the month of September, 1941, were cool and wet. During this period very little spread of crown rot occurred in inoculated trees, and the inoculations carried out yielded a very low percentage of crown rot lesions.

VARIETAL RESISTANCE

During the course of experiments designed to establish the pathogenicity of *P. cactorum*, trees of several varieties were inoculated with this organism. The results of these inoculations gave a sufficiently strong indication of the existence of varietal variations in resistance to crown rot to justify further investigations. Experiments carried out during the summers of 1940 and 1941 have given clear evidence that several varieties, notably the McIntosh, are characterized by consistent resistance to the disease, and several others, including the Canada Baldwin, by greater degrees of susceptibility. The seedlings derived from certain varieties have given characteristically resistant or characteristically susceptible performances. In these earlier experiments

a single isolate of *P. cactorum* has been used throughout. In co-operation with the Dominion Experimental Station at Summerland the tests are being expanded. Mixed inocula of several *P. cactorum* isolates are being employed as a safeguard against possible existence of strain differences within the species that might modify the varietal resistance picture.

The complete results of these crown rot resistance studies will be published at a later date.

ADDITIONAL FACTORS AFFECTING CROWN ROT INCIDENCE

TREE DORMANCY

In the Wisconsin tank experiments described above a greater degree of resistance was displayed by dormant trees throughout the ranges of soil moisture and temperature. Where dormant trees were attacked the appearance of the disease was delayed and the spread of the lesions was slow.

DEPTH LIMIT FOR DISEASE OCCURRENCE

Field observations have indicated a general restriction of crown rot spread to the portion of the crown that lies immediately beneath the surface of the soil. The lower limit of the rot is usually within six inches of the soil surface, and is very seldom found at a depth greater than nine inches.

In inoculation experiments this same restriction of the rot has been encountered. The experiment already outlined, as varietal resistance Experiment II, provided an interesting example. Here the inoculated trees were all of the McIntosh variety, growing on various seedling and Malling rootstocks. While in most cases the stock-scion junction was situated within several inches of the soil surface, in certain trees it was buried as deeply as eight inches. Since the McIntosh material appeared to be uniformly resistant, it was necessary that crown rot in these trees should appear below the deep junctions.

Of the three trees whose stock-scion junctions were about eight inches deep, crown rot appeared in only one, and in this tree the rot ceased its spread after involving less than one-quarter of the circumference of the crown. Of the five trees whose stock-scion junctions were about six inches deep, crown rot was initiated in all five, but in every case it ceased to spread before one-half of the crown had been involved. Of the 98 less deeply planted stocks included in the experiment, crown rot was initiated in 52, with a spread of the lesions over the entire crown in 26, and active spread continuing in many of the remaining trees at the time of the final observation.

WOUND ENTRY OF *P. cactorum*

In all but several of the 200 trees in which crown rot has been produced by exposure of their healthy crowns to inoculum of *P. cactorum*, the lesions have centred in bark wounds deliberately inserted at the beginning of the experiment. In the several trees in which the rot has centred elsewhere there has existed a possibility that accidental wounds were present to act as avenues of entry.

All these deliberately made wounds which acted as entry points for the fungus involved the removal of bark to the depth of the cambium. During the uncovering of the crowns of the trees for inoculation, numerous accidental wounds were made involving only the outer layers of bark. These superficial wounds were never known to act as centres for crown rot initiation.

RESTRICTION OF CROWN ROT SPREAD

When crown rot is once initiated the extent of the lesion varies from tree to tree. Probably in certain cases this is a function of changing soil conditions. However it has been noticed in the various inoculation experiments that the structure of the crown of the tree may determine the extent of the lesion spread. Frequently when crown rot is spreading over the portions of the crown from which roots arise the lesion encircles the root bases before any penetration of the root tissues occurs. For days, or even weeks, a clean margin will exist precisely at the junction of root and crown. A similar pause occurs in the spread of the rot from large roots into the secondary roots arising from them. Frequently a sound rootlet may be observed arising from a point on the diseased root which is several inches within the margin of the advancing lesion.

FACTORS RESPONSIBLE FOR THE APPARENT STERILITY OF CROWN ROT TISSUES

Two explanations have been considered for the difficulty experienced in isolating *P. cactorum* from crown rot tissues, even when microscopic examination has revealed the presence of mycelium. The first possibility is that saprophytes or secondary parasites, closely following the primary parasite through the tissues, are able to inhibit its growth in all but the marginal region; the second is that degradation products of the dying host tissues are toxic to the fungus. Experiments have been carried out to test these possibilities.

INHIBITION BY OTHER ORGANISMS ON NUTRIENT MEDIA

A test was made of the ability of four other crown rot isolates to affect the growth of *P. cactorum* in pure culture on potato dextrose agar. The organisms used were bacteria isolated in the summer of 1940. In each test the four bacteria were planted on a Petri plate at equal distances from a central planting of *P. cactorum* isolate No. 1. The plates were placed in an oven at 25° C.

Identical results were obtained in the six plates used. After two days the growth of the fungus colony was arrested 13 mm. from the margin of the colony of bacterial isolate No. 2. After three days a similar halting of fungus growth was effected 10 mm. from the margin of the colony of isolate No. 3. The degrees of inhibition effected by the four bacterial isolates after eight days are illustrated in Fig. 5. It may be noted that bacterial isolate No. 1 is the organism listed in the writer's stock culture catalogue as isolate 452b, which arises most frequently from plantings of crown rot tissue.

PLATE III

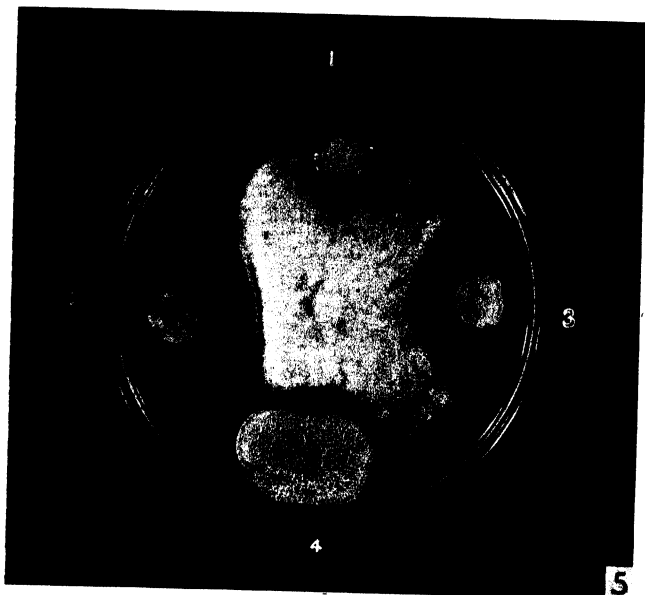
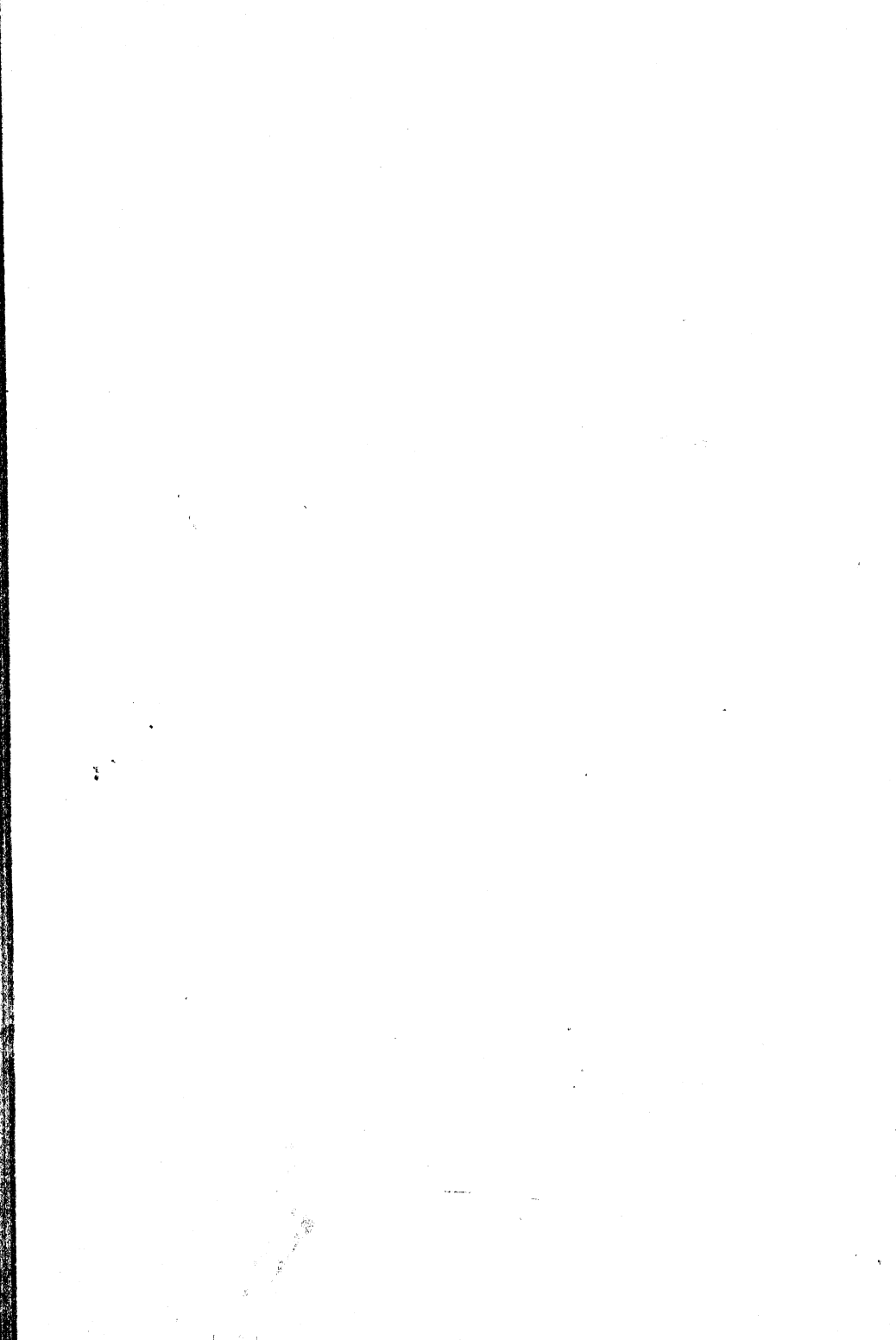


FIG. 5. *The influence on colony growth of *Phytophthora cactorum* of four bacterial isolates from crown rot tissues. Photographed after eight days' growth on potato dextrose agar at 25° C.*



In liquid nutrient media it has been noticed repeatedly that *P. cactorum* will cease growth whenever bacterial contaminants gain entry. The bacterial isolate 452b which so commonly arises from plantings of crown rot tissue possesses this ability to suppress growth of the fungus.

THE PURE CULTURE OF *P. cactorum* IN BARK TISSUES

Efforts have been made to produce a rotting of apple bark tissue under conditions in which all organisms except *P. cactorum* could be excluded. This has required a sterilization of living bark tissue and a subsequent inoculation of this tissue with the fungus. Several methods were tested and found unsuitable, either because they gave imperfect sterilization or because they caused injury to the bark tissue. Finally an experimental procedure was devised which provided a measure of success. One-inch lengths of crown tissue from vigorous young apple trees were used as test material. This material was sterilized by a slight modification of the acetic acid method for wood block sterilization described by Fritz (5), the times of washing being approximately doubled. This sterilized material was exposed then to inoculum of *P. cactorum* in sterile moisture chambers. At the end of 22 days the bark tissues were brown and rotten. Microscopic examination of tissue scrapings revealed the presence of mycelium throughout. Oogonia, antheridia, and oospores were present in large numbers. *P. cactorum* was isolated readily from the rotted tissue. Check material, sterilized in the same manner but not exposed to inoculum of the fungus, remained sound. Microscopic examination revealed little or no plasmolysis of the cells and an absence of fungal mycelium. Tissue plantings on nutrient media remained sterile.

THE DEMONSTRATION OF *P. cactorum* IN INFESTED SOIL

Investigation of certain phases of the epidemiology of crown rot, particularly of the persistence and activity of *P. cactorum* in the soil under varying conditions of environment and culture, has awaited the development of some method that would allow ready detection of the presence of the fungus in soils. Standard methods have proved useless for the isolation of *P. cactorum* either from naturally-infested or from artificially-infested soils. The apple fruit method of Tucker (27) on several occasions has yielded the fungus from artificially-infested soil, but never from orchard soils about crown rotted trees, since the fruits invariably are rotted very rapidly by other soil-inhabiting fungi.

It was believed that a susceptible, easily grown host plant might provide a sufficiently specific substrate for the detection of *P. cactorum* in infested soils. Of the plants that have been reported as susceptibles, the several selected as most suitable for this type of experiment were *Salpiglossis*, *Eschscholtzia*, *Godehia*, the garden pepper, *Capsicum annuum*, and the sugar beet. Seed was obtained of all these plants and tests were made of their susceptibility to inoculum of *P. cactorum* in sterilized soil.

The *Salpiglossis*, *Eschscholtzia*, and sugar beet suffered damping-off. However the *Salpiglossis* and *Eschscholtzia* seedlings germinated slowly, emerging 10 days after sowing, and they were too small to allow ready isolation of *P. cactorum*. Crushed mounts revealed the presence of oogonia and antheridia of the fungus within the seedling tissues. The sugar beet seedlings emerged after four days, and damping-off appeared two days later. *P. cactorum* could be obtained from plantings of diseased tissue after a surface sterilization with 1 : 1500 mercuric chloride for one minute.

A further test was made of the effectiveness of a sugar beet catch-planting for isolation of *P. cactorum*, this time using infested unsterilized soil. Unfortunately this revealed that the sugar beet suffered damping-off more readily from other pythiaceae fungi than from *P. cactorum*, and was therefore unsuitable.

There remains some justification for further testing of the suitability of *Salpiglossis* seedlings as a selective substrate. The difficulty of isolating the fungus from them would not be a serious drawback as long as the fruiting structures were produced readily in the diseased tissues.

COMPARISON OF *P. cactorum* ISOLATES FROM INDIANA TRUNK CANCER AND BRITISH COLUMBIA CROWN ROT

Baines (1, 2) has demonstrated that *P. cactorum* is the causal agent of a trunk canker of apple trees in Indiana and neighbouring states. Although this disease and British Columbia crown rot are essentially similar apple bark rots and are caused by the same species of *Phytophthora*, the two diseases appear to differ in the varieties and ages of trees attacked, and in the locus of attack in susceptible trees.

Dr. Baines was kind enough to supply the writer with a transfer of his culture No. 1, isolated from an apple tree trunk canker in 1935. Limited comparisons have been made of certain morphological and physiological characters, as well as of the pathogenic capabilities of this trunk canker isolate and crown rot isolate No. 1, in an attempt to determine whether the points of variance of the two diseases are due to strain differences in the causal organism.

COLONY CHARACTERISTICS AND MORPHOLOGY

Comparisons were made of the colony characteristics of the two isolates and of their ability to produce reproductive structures when grown on potato dextrose agar, oatmeal agar, and Leonian's malt extract-yeast extract agar. On none of these media were significant differences displayed.

Examination and measurements were made of the reproductive structures produced by the two isolates when inoculum was inserted into ripe apple fruits and the resulting rotted tissue transferred to sterile distilled water. The mean dimensions of 20 oogonia of the trunk canker isolates were $23.7 \mu \times 22.8 \mu$, and of the crown rot isolate $23.9 \mu \times 22.8 \mu$. The sporangia of both isolates were distinctly papillate. The antheridia were predominately paragynous.

GERMINATION OF SPORANGIA

Causal observation has indicated that the bulk of the sporangial germination of both isolates in culture is by germ tube. However, germination by swarm spore emission was induced by the flooding of 14-day old colonies with sterile distilled water and the storing of the sporangium suspensions at 60° F. for a period of two hours. Of 55 sporangia of the trunk canker isolate which were examined after such treatment, 23 germinated by germ tube, 31 by swarm spore emission, and one by the production of a secondary sporangium. Of 55 sporangia of the crown rot isolate, 30 germinated by germ tubes, 19 by swarm spore emission, and six by secondary sporangia.

TEMPERATURE RELATIONS IN PURE CULTURE

The temperature-growth relations of the trunk canker isolate and the crown rot isolate were tested in parallel series on Leonian's malt extract-yeast extract agar. The experiment was carried out in triplicate, for a period of eight days, with daily measurements of colony diameter. The temperature-growth curves of the two isolates are compared in Fig. 6. Their growth was

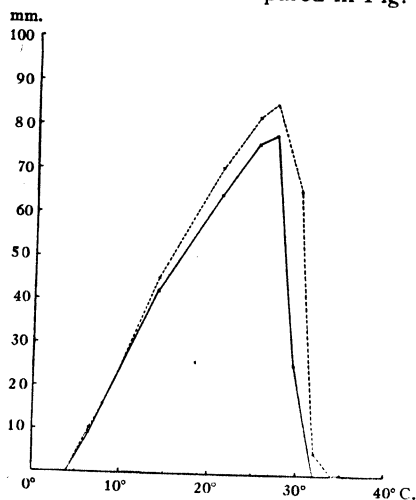


FIG. 6. Comparison of the temperature-growth relations on potato dextrose agar of *Phytophthora cactorum* isolate No. 1 from crown rot and Baines' isolate No. 1 from Indiana trunk canker; —●—●— = trunk canker isolate, ---+---+--- = crown rot isolate.

essentially the same at all temperatures below 30° C. At this temperature the growth of the trunk canker isolate was slower and more irregular than that of the crown rot isolate. At 32° C. the former was incapable of even the weak growth put forth by the crown rot isolate at this temperature.

Experiment I

PATHOGENICITY

The pathogenicity of the two isolates was compared on two-year old apple trees in the greenhouse. Five trees each of the varieties Canada Baldwin

and Grimes Golden were used in the test of each isolate. The trees were an assorted group, varying in stage of development from dormancy to active terminal growth, all however having been introduced to the greenhouse at least four weeks before the organization of the experiment. The trees were pruned and potted in the same manner as those destined for the temperature tank experiments described earlier in this paper. The soil in the upper one-quarter of each can was infested with the isolate under test, grown on corn-meal, sand, canned pea mixture. The crowns of the trees were wounded by the removal of strips of bark tissue. The soil moisture level was maintained close to saturation. The soil temperature fluctuated between 21 and 27° C. Atmospheric humidity remained at about 30% of saturation. The results were as follows.

Trees inoculated with the trunk canker isolate:

- Canada Baldwin—5 trees remained disease-free.
- Grimes Golden— 1 tree girdled.
2 trees partially girdled.
2 trees remained disease-free.

Trees inoculated with the crown rot isolate:

- Canada Baldwin—2 trees girdled.
3 trees remained disease-free.
- Grimes Golden— 1 tree girdled.
1 tree partially girdled.
3 trees remained disease-free.

Experiment II

This experiment was carried out under the same conditions as Experiment I. It was designed as a further test of the pathogenicity of the trunk canker isolate in the Canada Baldwin variety. The 10 Canada Baldwin trees used were healthy and in full leaf at the time the experiment was initiated. The trunk canker isolate was introduced into the surface soil in corn-meal-sand medium. The crowns of the trees were wounded in the usual manner. The soil moisture was maintained close to saturation.

After 14 days one tree was girdled at ground level. A second tree was one-half girdled, also at ground level, after 20 days. The lesion had ceased its spread at this time. The vigour of both of these trees had declined noticeably before the disease appeared. In one tree all the leaves were scorched and killed, and in the other many of the leaves had suffered the same fate. No disease was produced in the trees whose vigour remained unimpaired.

Twenty-two days after this inoculation with the trunk canker isolate the crowns of the eight trees remaining disease-free were wounded again, and inoculum of the crown rot isolate was added to the surface soil about the crowns of four. Within 20 days two of the four trees inoculated with the crown rot isolate had been girdled. These two trees were growing vigorously when the disease appeared. The four trees that were not exposed to inoculum of the crown rot isolate remained healthy.

The results of these two experiments combine to suggest that the trunk canker isolate is less readily pathogenic on the Canada Baldwin variety than is the crown rot isolate. Some indication is also given that under conditions in which the crown rot isolate produces below-ground symptoms the trunk canker isolate produces symptoms in the above-ground tissues of Grimes Golden. Unfortunately the numbers of available trees were so small that the results remain doubtfully significant.

Discussion

THE DEMONSTRATION THAT CROWN ROT IS A PARASITIC DISEASE

For many years crown rot has been classified as a physiological disorder. In British Columbia it was believed to be the direct result of the subjection of trees to conditions of excess soil moisture. This conception was formed only after an extensive search for a causal organism had proved unsuccessful. Crown rot tissues had not yielded any single fungus or bacterium with sufficient consistency to justify its consideration as a causal agent of the disease.

It now appears clear that crown rot is a parasitic disease whose occurrence is determined by a number of secondary factors, of which the soil moisture factor is the most obviously effective. The causal fungus *P. cactorum* can be isolated from crown rot tissues, but only with considerable difficulty. The most convincing proof of the parasitic nature of the disease has been provided by the demonstrated ability of *P. cactorum* to reproduce typical symptoms of the disease in inoculated trees rather than by the consistency with which the fungus can be isolated from rotted tissues. Inoculations with *P. cactorum* have resulted in the production of typical crown rot symptoms in over 100 apple trees in the field and in a further 93 trees in greenhouse experiments.

The success of these pathogenicity tests has given adequate proof of the ability of *P. cactorum* to produce crown rot symptoms. The broader conclusion that *P. cactorum* is responsible for the general occurrence of the disease in British Columbia has been justified by certain additional evidence. The identity of the disease wherever found has been amply demonstrated by the presence of characteristic fungal mycelium throughout freshly rotted tissues, whether the lesions from which these tissues are collected have resulted from natural infection or from inoculation with *P. cactorum*, and whether or not *P. cactorum* can be isolated from them.

The isolation results have been consistent in that *P. cactorum* has been yielded only by tissues at the margin of active crown rot lesions. It is evident that the activity of *P. cactorum* is inhibited in some manner in the tissues behind this margin. Usually tissues immediately behind the margin of a lesion yield the bacterial isolate designated 452b. The possibility has suggested itself therefore that *P. cactorum* is closely followed in its advance through the tissues by the bacterium, which behaves as a saprophyte or secondary parasite, and which has the ability to inhibit further growth of the primary parasite. Thus the activity of *P. cactorum* would be limited to those tissues

at the margins of the lesion, whose organization had not been sufficiently broken down to allow penetration of the secondary organism.

This theory has been supported by the striking susceptibility of *P. cactorum* to inhibition by bacterial contaminants including the bacterial isolate 452b. The culture experiments have indicated that, although the inhibitory effect of this organism on *P. cactorum* on potato dextrose agar is not as striking as that of certain other bacteria, the fungus is unable to overgrow colonies of isolate 452b. In liquid media the introduction of isolate 452b immediately arrests the growth of *P. cactorum*.

It must be noted that occasionally crown rot tissues containing the characteristic mycelium will yield neither *P. cactorum* nor bacterial growths. In these tissues it must be presumed either that both organisms have died or that the inhibition of *P. cactorum* is due to some factor other than bacterial inhibition.

The degradation products of dying host tissue must be considered as possible alternative inhibitors of *P. cactorum*. No indications of such inhibition have been encountered in the course of the investigation. On the other hand there is some negative evidence. When *P. cactorum* was cultured in sterile bark tissues in the laboratory the fungus was available for isolation from tissues that had been rotted for two weeks, and that had assumed the appearance of old inactive lesions. Moreover fruiting bodies of the fungus were produced in abundance in these tissues, whereas they have never been observed in the crown rot tissues collected from naturally-occurring crown rot or that produced by inoculation with *P. cactorum*.

THE EPIDEMIOLOGY OF CROWN ROT

The demonstration that *P. cactorum* is the causal agent of crown rot has afforded an opportunity to produce the disease more or less at will, and thus has made possible a more thorough study of the factors influencing its occurrence. Before attempting to interpret the occurrence of the disease in terms of these various factors it seems advisable to consider separately the data that have been obtained on the operation of several of these factors.

THE INFLUENCE OF SOIL MOISTURE AND SOIL TEMPERATURE

The demonstration that crown rot is a parasitic disease has not minimized the importance of soil moisture as a factor governing its occurrence. The correlation of disease occurrence with conditions of excess soil moisture was established beyond doubt by the exhaustive and careful field observations of Roger (20).

Controlled experiments in the Wisconsin tanks have agreed with the field observations in demonstrating that high soil moisture favours the disease. Under the conditions of experiment however this was only true at higher temperatures, above 23° C. in the Canada Baldwin variety, above 17° C. in the Grimes Golden. It is believed that the results of tests using Canada

Baldwin are the more reliable throughout because of the interference of the dormancy factor in Grimes Golden. The importance of the soil moisture factor reached its peak at 32° C. where reduction of soil moisture from 96% of saturation to 60% reduced the severity of crown rot attack from 100% to zero. In moist soil at this temperature the crown rot attack was more rapid than under any other conditions. Moreover it approximates the surface soil temperature at which the inoculation of trees in the field has been most successful.

The combined evidence from field observations and the experiments in Wisconsin tanks indicates that subsoil moisture exerts a more important influence than the moisture level of the surface soil immediately surrounding the crown of the tree. It appears then that soil moisture does not exert its principal effect in the locus of crown rot attack, but operates rather as a factor predisposing the tree to attack. The Wisconsin tank experiments offered some evidence that the nature of this predisposition was a reduction of the vigour of the tree. Although the disease appeared in trees whose vigour had not suffered such impairment, increased susceptibility was evident in trees whose root systems had suffered killing in the near saturated soil. No evidence was obtained to support the alternative possibility, that the high moisture level increased susceptibility by increasing the succulence of susceptible tissues.

Although crown rot can occur when the moisture level of the surface soil is relatively low, surface soil moisture cannot be ignored entirely as a contributing influence. The restriction of crown rot to below-ground tissues is best explained as the expression of a moisture requirement. Moreover the established practice of arresting the spread of crown rot lesions by unearthing the attacked crowns is believed to depend on the exposure of the tissues to low atmospheric humidities. In field inoculation experiments there was a suggestion that crown rot initiation and spread were discouraged when surface soil moisture levels fell below 25% of saturation.

A two-fold effect of soil temperature seems probable: a predisposing effect on the host, and a direct effect on the activity of *P. cactorum*. Its role in predisposition was evidenced in the Wisconsin tank experiments when the imposition of excess soil moisture conditions at high temperatures caused a reduction of tree vigour by root killing. Throughout the range of temperatures below 29° C. the incidence of disease corresponds closely to the temperature response of the causal fungus in pure culture.

The demonstrated inability of *P. cactorum* to survive desiccation in culture suggests that, unless the fungus is able under certain conditions to produce special resistant structures, it may be able to survive only in soils and tissues whose moisture level remains high.

VARIETAL RESISTANCE

In British Columbia there has always existed some uncertainty over the reality of varietal resistance to crown rot. Data from observations in commercial plantings have been undependable because of the existence in every

tree of two genetically distinct entities, the scion and the rootstock, usually with portions of both included within the locus of crown rot attack. The rootstocks used are seedlings, usually of unknown origin. This difficulty has been overcome in varietal resistance experiments by the use of trees in which the origin of both scion and rootstock is known, and the location of the stock-scion junction apparent. The varieties already tested in such experiments have given clear evidence of differing degrees of resistance to crown rot, indicating that varietal resistance is an important determinant of crown rot incidence.

GENERAL CONSIDERATION

Many vagaries of crown rot occurrence in the Okanagan Valley which are not explainable on the basis of soil moisture correlations alone, can now be accounted for much more readily.

Evidence that varieties vary in their degree of resistance and that the resistance of a variety is plastic, subject in particular to modification by soil moisture and temperature, offers a plausible explanation for the frequent but not invariable correlation of crown rot outbreaks with conditions of excess soil moisture. It seems probable that the prerequisites of a serious outbreak are essentially the exposure of susceptible varieties or of trees growing on susceptible rootstocks to conditions of excess subsoil moisture, and the provision of wounds to serve as avenues of entry for the causal organism.

The restriction of crown rot initiation and spread to the summer months may be ascribed both to the influence of temperature on host and pathogen, and to the increased resistance of dormant trees.

The limitation of crown rot to tissues within several inches of the surface of the soil is important where stock-scion junctions are deeply placed. It is believed that an aeration requirement of the fungus restricts its activity at greater depths. The limitation of lesion margins by morphological peculiarities of the host is important when large roots arise from the crown near the soil surface. Not infrequently the rot is confined to a single sector of the crown by its inability to penetrate from crown tissues into those of the root. The nature of this morphologic barrier at the base of a root is unknown.

Before any of the predisposing factors can be effective it is of course essential that *P. cactorum* be present in the orchard soil. The lack of a method for satisfactory demonstration of the presence of the fungus in soils has prevented any direct study of the factors influencing its persistence and activity outside the tissues of the host. A limited amount of circumstantial evidence exists to indicate that the fungus is not present in all Okanagan orchard soils at all times. This was suggested in particular by the fact that a high percentage of success was obtained in the initiation of crown rot by applying inoculum of *P. cactorum* to wounds on the crowns of seven-year old trees in two orchards that had not suffered crown rot attack previously. Since the soil conditions were not altered, the success obtained must have been a result either of the wounding of the crowns or of the introduction of inoculum into the surrounding soil. Regular disking in both orchards must have inflicted numerous wounds.

Therefore it is probable that conditions were unsuitable for the persistence of *P. cactorum* in the soil. The performance of *P. cactorum* in culture suggests that it has a low resistance to desiccation, and raises the possibility that it will not persist in dry soils. The general confinement of crown rot in British Columbia and the Pacific Northwest to regions under irrigation has suggested the interesting possibility that irrigation water serves as a distributing agent for inoculum of the causal fungus. The recent demonstration by Blackwell *et al.* (4) that species of *Phytophthora*, including *P. cactorum*, are able to live as water moulds, suggests further that the fungus might live and multiply in reservoirs and be carried into orchard soils each year by the water therefrom. The operation of such a factor would be difficult to demonstrate.

The secondary above-ground symptoms of crown rot appear to be a direct result of the separation of the root from the foliar portions of the tree. A more rapid induction by *P. cactorum* of secondary symptoms through the liberation of a mycotoxin into the vascular tissues, recently demonstrated in the case of the bleeding canker of hardwoods by Howard (13), does not appear to occur in apple tree crown rot, since mechanical injuries result in identical above-ground symptoms after a similar lapse of time.

CROWN ROT IN THE LITERATURE

This investigation has been concerned particularly with crown rot as it occurs in British Columbia orchards. The names "crown rot" and "collar rot" however have been employed by workers in most of the apple-growing regions of North America to characterize the forms of trunk and crown injury encountered in those regions. It is clear that a group of distinct disorders has been assembled under these two names. The locus of attack has been variously reported as above-ground, below-ground, or both. The causes ascribed have been even more varied. However the similarity of the described symptoms and conditions of occurrence of the crown disease in certain other regions suggests that the British Columbia form of the disease may have a wider occurrence.

Crown rot has received considerable attention in the Wenatchee and Yakima valleys of the state of Washington. These regions form a fairly continuous fruit belt, of which the Canadian portion of the Okanagan forms the northermost extreme. Since the great bulk of this entire belt is under irrigation, varieties and orchard practices remain reasonably uniform throughout. Hotson (12), Heald (10), and Magness (17) have studied and described collar rot in the irrigated districts of Washington. Hotson contends that the initial injury is produced by any one of several factors, including fire-blight, rodent injury, winter injury, arsenical injury, mechanical injury, and defective bark unions, but that the healing of the wounds is prevented by the presence of excess moisture and other factors. Heald, on the basis of field observations, concludes that crown rot is primarily a form of winter injury. Magness adheres to the winter injury theory, providing data to illustrate the susceptibility of crown tissues to cold temperature injury. Magness gives a descrip-

tion of symptoms that correspond splendidly with those of crown rot in British Columbia. He implied that the collar rot referred to by the three workers is the same. The locus of attack is essentially the portion of the crown and roots immediately below ground level. All three workers mention an apparent spread of the rot beyond the margins of those injuries that they conceive to be primary. Hotson and Magness note the frequent concurrence of the disease and excess soil moisture conditions, but consider the exceptions to be sufficiently frequent to outlaw excess moisture as a primary factor.

Crown rot conditions have been reported from others of the western states. Ball *et al.* (3) report the occurrence of a crown rot in orchards of Utah and Colorado which they consider to be identical with the condition observed during a survey of the Wenatchee and Yakima valleys. Ball in Utah, and Paddock (18) and Headden (8, 9) in Colorado recognize several crown rot conditions in the two states, ascribing them variously to arsenical residues, alkali, seepage conditions, and excess of nitrates, with Ball and Headden disagreeing on the relative importance of these various factors.

Grossenbacher (6, 7) and Thomas (25) have described crown rot conditions occurring in New York, with the locus of attack sometimes above and sometimes below ground level. Both workers ascribe the bulk of the injury to frost killing. Although Thomas and MacDaniels in a later paper (26) point out that the condition that they have described is entirely the result of winter killing, with healing of the injuries during the ensuing season whenever sufficient vigour remains in the trees, Grossenbacher reports having observed instances of lesion spread during the summer months.

The collar rot or trunk canker reported from the apple orchards of the Middle West is essentially an above-ground disease. Baines (2) has adequately reviewed the various reports of this trouble, from workers in Michigan, Ohio, Indiana, and Illinois, as well as New York and Pennsylvania. Baines presents a clear account of the occurrence and symptoms of the disease in Indiana. The nature of the tissue decay differs little from the British Columbia disease. There are several points of distinct variance between the two diseases however. In particular the Indiana cankers occur mainly on the trunk and scaffold branches, only occasionally invading the below-ground portions of the trees. The disease in Indiana is confined almost entirely to the Grimes Golden variety. Varieties such as Winesap, Northern Spy, and Jonathan which frequently suffer from crown rot in British Columbia are listed as resistant. The Indiana disease seldom occurs in trees under 13 years of age, whereas British Columbia crown rot is found consistently in trees of all ages from one year to 40. The soil moisture factor which is so important in determining disease occurrence in British Columbia has not been mentioned as operative in the regions of trunk canker occurrence.

The Indiana trunk canker is particularly interesting, because Baines (1) has demonstrated that *P. cactorum* is the causal agent of this disease. The tissues rotted by trunk canker appear to have yielded *P. cactorum* much more

readily than have crown rot tissues. The isolates that he obtained were pathogenic on a total of seven varieties, but he lists among the varieties that proved resistant to his isolates, several that are attacked by the crown rot isolate. The symptoms resulting from inoculation of trees with his isolates, like the symptoms of the disease from which they were obtained, were confined to above-ground tissues. Little success was obtained in the inoculation of trees under 13 years of age.

Thus the same species of fungus has been demonstrated as the causal agent of two apple bark rot diseases, with the locus of attack shifted from above-ground tissues in the one case to below-ground tissues in the other, and with striking differences in the varieties and ages of trees attacked.

The origin of these peculiarities of the two diseases offers an interesting problem. The difference in locus of attack might be attributed to three separate factors. Baines implies that the restriction of cankers to above-ground tissues in Indiana can be attributed to the resistance of the French Crab seedling rootstocks on which the trees are grown. In British Columbia there is on the other hand a diversity of rootstocks and considerable variation in depth of planting. However this does not explain the rare occurrence of above-ground cankers in British Columbia. This must be a function either of climatic conditions or of strain differences in *P. cactorum*. The pathogenicity comparisons of the two isolates in the greenhouse suggested the latter, but in view of the small numbers of trees used the results were not convincing.

The discrepancies in the degree of resistance of certain apple varieties to the two diseases are most plausibly explained by strain differences within the species *P. cactorum*. Pathogenic strains of this species have been demonstrated by several workers. Baines has obtained evidence of strain differences among the isolates yielded from the trunk canker disease. Comparison by the writer of isolates from the two diseases revealed no significant differences in colony characters or morphology. There was however a noticeable difference in the response of the two isolates to temperature levels above 27° C. The greenhouse pathogenicity tests suggested that the Canada Baldwin variety is less susceptible to the trunk canker isolate than to the crown rot isolate.

THE CONTROL OF CROWN ROT

The methods now used to combat crown rot in British Columbia orchards have been designed to take advantage of two known facts: first that crown rot is usually most severe when soil moisture is high, and second that the disease thrives only in tissues below the surface of the soil. The following are the principal recommendations:

- (1) An examination of the crowns of all suspected trees in late summer.
- (2) The uncovering in early spring of the crowns of all trees in which the disease has been noted, and the removal of all rotted bark tissue. The diseased crowns should be allowed to remain exposed until late fall, when the danger of winter injury requires the replacement of the removed soil.

(3) The removal of girdled trees, and aid to the recovery of partially girdled trees by the inarching of suckers or young seedling trees above the scarified lesions.

(4) The reduction of irrigation applications to the minimum requirements of the trees themselves, combined with a correction of seepage conditions where they occur, and the drainage of low-lying orchard land.

The application of these recommendations usually has resulted in the recovery of attacked trees and the discouragement of further occurrences of the disease. However there have been encountered from time to time orchards in which the occurrence of crown rot cannot be correlated with moisture excess, and other orchards for which reduction of moisture supply has proved difficult or impossible. The practices of excavation and surgery prove time-consuming and laborious when conditions make it necessary that they be repeated year after year. Thus there remains a need for the development of improved methods of combating the disease.

The present investigations have verified the soundness of the moisture reduction recommendations, and have placed emphasis on the importance of reducing subsoil moisture, even where the moisture level of the surface soil is low. They have stressed the particular importance of soil moisture regulation during the hot summer months. The control recommendations must now be accompanied by a warning of the danger of inflicting wounds in the buried portions of the crown.

In addition, two entirely new methods of combating crown rot have been suggested as a result of the additional knowledge of its nature and epidemiology. The disclosure that the disease is caused by a soil pathogen has led logically to an investigation of the efficacy of the applications of soil fungicides. The demonstration of varietal resistance to the disease has made possible an extensive search for resistant varieties and rootstocks.

THE USE OF SOIL FUNGICIDES

Elimination of the causal organism from the soil surrounding apple tree crowns by the application of fungicides is an attractive possibility. The absence of sensitive rootlets in the immediate vicinity of the locus of disease attack should allow the application of the chemicals in much higher concentrations than those that can be applied to herbaceous crops.

Copper sulphate has been selected as the first test material, since it is cheap, is an effective fungicide, and can be applied in relatively large quantities without poisoning the soil. Copper sprays and damping-off treatments have proved effective against *Phytophthora* species, and Skaptason *et al.* (22) have suggested that large residual quantities of copper in Long Island soils have reduced the incidence of tuber rot of potatoes by an inhibition of the sporangial germination of *Phytophthora infestans*.

The tests that are being made of this compound should provide an indication both of its effectiveness in protecting the trees from crown rot attack and of

any harmful effects which may be produced by its applications about the crowns of the trees.

THE EMPLOYMENT OF RESISTANT VARIETIES AND ROOTSTOCKS

An ideal eventual solution of the crown rot problem would be attained if entirely resistant material could be employed in future apple plantings. The practicability of such a solution has been indicated by the results obtained thus far in varietal resistance tests. An extensive search for resistant varieties and rootstocks is now in progress, using as material a large selection of seedling rootstocks, Malling rootstocks, and framework stocks which has been assembled for tests of general orchard performance by the horticulturists of the Dominion Experimental Station at Summerland. Since the use of standard breeding methods would not be feasible for a heterozygous and slow-growing host such as the apple, a resort is necessary to this wholesale testing of diverse material, with selection of that which proves most resistant. It is especially desirable that the crown rot resistance trials be made first in material already recommended by the horticulturist.

Acknowledgments

The field studies of crown rot have been carried out at the Dominion Laboratory of Plant Pathology, Summerland, B.C. Dr. R. E. Fitzpatrick suggested the problem and has maintained an active interest throughout. Dr. H. R. McLarty as Director of the Laboratory has been generous with advice and encouragement. The trees for the field tests have been provided through the kindness of Mr. R. C. Palmer and Mr. A. J. Mann of the Dominion Experimental Station at Summerland.

The laboratory and greenhouse studies have been carried out in the Department of Botany, University of Toronto, and have profited greatly from the supervision of Dr. D. L. Bailey. A Studentship and Fellowship from the National Research Council have made possible these phases of the investigation. The trees used have been provided by the Department of Botany.

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Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 20, SEC. C.

OCTOBER, 1942

NUMBER 10

RELATION OF LOOSE SMUT TO YIELD OF BARLEY¹

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Abstract

A simple technique was devised to inoculate barley with *Ustilago nuda* (Jens.) K. and S. in order to facilitate the study of the effect of loose smut on yield. Infected seed lots so obtained were mixed in different proportions with normal seed to provide at least six levels of infection ranging from nearly zero to the maximum. The infection levels were planted in replicated field test plots at three stations in each of two years.

No significant correlations were obtained between yield of barley and the percentage loose smut in the 1939 trials. A direct linear relationship was obtained in 1940 between percentage incidence of loose smut and the reduction in yield. For every 1% increase in loose smut, yield was reduced 0.85% at St. Paul, Minn., 1.4% at Edmonton, and 1.2% at Fallis, Alta. These regression coefficients are not significantly different. Loose smut did not affect tillering noticeably. Similar infections of loose smut were obtained at all stations for comparable seed lots. Higher infections were observed as the rate of seeding was increased. The incidence of loose smut at Edmonton was the same on the basis of percentage smutted spikes as on the basis of percentage smutted plants.

Introduction

Loose smut of barley, caused by *Ustilago nuda* (Jens.) K. and S., is a very common disease in the field. Many fields are recorded showing 10% loose smut while a few fields have shown even 50% (1). The reduction in yield from this disease has not been determined but a high positive correlation has been assumed.

Previous attempts to determine the reduction in yield of barley caused by loose smut were rendered difficult through insufficient quantities of seed exhibiting high percentages of the fungus. Commercial seed samples proved unsatisfactory for this purpose because of their low percentages of loose smut and their likelihood of containing varietal mixtures. A simple technique for inoculating barley in the field with *U. nuda* was devised to provide adequate quantities of seed of known varietal purity, normal size and appearance, and which on planting exhibited a high percentage of loose smut. Such seed was mixed with different proportions of normal healthy seed to make samples showing different percentage levels of diseased seed. The samples so obtained were planted in replicated field trials at several stations from which the relationship of yield to the percentage of loose smut was determined.

¹ Manuscript received in original form January 31, 1942, and as revised, July 13, 1942.

Contribution from the Department of Field Crops, University of Alberta, Edmonton, Alta., with financial assistance from the National Research Council of Canada.

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Experimental

Material and Methods

Newal barley (C.A.N. 1089*) obtained from certified seed stock of the University of Alberta, was the variety used in the present study. This variety had shown as high as 5% loose smut in commercial fields and averaged 35% over a three year period of artificial inoculation in field plots.

Method of Inoculation

Artificially infected seed lots of this variety were produced in Edmonton during the two years, 1938 and 1939, previous to their use in yield test plots. Inoculum of *U. nuda* consisted of chlamydospores obtained from a large composite collection of infected barley spikes. These spikes were collected during 1938 from the Uniform Barley Nursery and the rod row trials of the Edmonton station and from commercial barley fields in and around Edmonton whereas in 1939 they were collected from the loose smut yield trial at the Edmonton station. The spikes were spread out in the laboratory and allowed to dry overnight at room temperature after which they were ground with a meat chopper and passed through a 144 mesh fly screen. Sifted inoculum was then placed in a four layer cheese-cloth bag from which chlamydospores were dusted by hand over the developing barley heads. Inoculation of the barley flowers in the field began when the barley heads first emerged from the boot in early July. The chlamydospores of *U. nuda* were dusted heavily over each spike by jarring the bag of spores against each head. Such dustings were made each evening over a two week period until all but the very latest tillers had headed. In this procedure, each head was dusted each evening for the duration of the two week period, and in all, four quarts of chlamydospores were used during the season on approximately 80 sq. yd. of grain. At harvest time the youngest and, therefore, uninoculated spikes were pulled out and discarded. Ten pounds of infected Newal barley seed were obtained in 1938 and 20 lb. in 1939. Greenhouse tests of the infected seed lots produced in 1938 and 1939 revealed 15.5 and 33.0% loose smut, respectively.

Preparation of Seed for Yield Trials

Taylor (3) found that smaller wheat seed segregates show higher percentage of infection with *U. tritici* than larger seeds. Accordingly the bulk sample of infected barley produced in 1938 for the 1939 yield tests was separated into four grades of which only the intermediate size, weighing 35.0 gm. per 1000 kernels as compared to 39.2 gm. for the bulk lot, was used for the yield tests in 1939. The infected seed lot produced in 1939 weighed 37.5 gm. per 1000 kernels compared with 42.1 gm. for a similar non-infected seed lot. No separation of seed was made for the 1940 yield test. The infected seed lots were divided and "diluted" with normal non-infected seed of the same variety to obtain samples yielding different amounts of loose smut. Six levels of loose smut percentage were used at all stations in 1939, when the mean maximum percentage of loose smut in the crop was about 11%, whereas in 1940, when the mean maximum of loose smut was 32%, six and nine levels were used.

* Canadian Accession Number.

Design of Experiment

Yield trials were conducted in 1939 at Fallis, Castor, and Edmonton, Alta. (the latter station, however, producing no yield data owing to hail damage) and in 1940 at Edmonton and Fallis, Alta., and St. Paul, Minn. The soil types at these stations were as follows: black loam at Edmonton, gray wooded loam at Fallis (5), silt loam on the fringe of the dark brown soil zone at Castor (4), and Waukegan silt loam at St. Paul. A 6 × 6 Latin square arrangement was used at all stations in 1939 and at the two Alberta stations in 1940. A randomized block arrangement with six replications was used at St. Paul. The treatment rows of infected seeds were separated by a row of non-infected seeds. In every case the rows were 18 ft. long and one foot apart, except at Fallis and Edmonton in 1940, where the rows were seven inches apart. Fourteen grams of seed (or approximately 370 seeds) were drilled in each row in 1939, while 400 seeds were sown in each row in 1940.

Data were taken for each plot on the number of infected and non-infected tillers and yield in grams. In 1939 the infected spikes were clipped off and recorded as they emerged from the boot, but in 1940, they were left intact and recorded only after the plot was cut at harvest. Seed producing tillers only were considered in determining the number of non-infected tillers. Precautions were taken against loss of seed in handling the harvested rows by wrapping the sheaves in cloth and threshing by hand in a muslin bag.

Results

The 1939 Yield Trials

The results from the 1939 yield trials at Castor and Fallis are shown respectively in Tables I and II. In these tables and those that follow, only the means of the replications are presented because such means were found to yield the same general result in calculation of the regression of yield on percentage loose smut as did the values for the individual plots.

TABLE I

MEANS OF SIX REPLICATES FOR PERCENTAGE LOOSE SMUT, TOTAL NUMBER OF SPIKES, AND YIELD IN GRAMS OF SIX LEVELS OF INFECTION WITH *Ustilago nuda* ON NEWAL BARLEY GROWN AT FALLIS, ALTA., IN 1939

Treatment	Percentage loose smut	Total number of spikes*	Yield*, gm.
Infected seed	10.8	299	301
4 Parts infected/1 part non-infected	9.4	310	317
3 Parts infected/2 parts non-infected	5.8	303	314
2 Parts infected/3 parts non-infected	4.0	320	340
1 Part infected/4 parts non-infected	2.3	309	322
Non-infected seed	0.1	327	337

* F value obtained for 5 D.f. for treatment and 20 D.f. for error is 0.78 for total number of spikes and 1.5 for yield, both being non-significant.

TABLE II

MEANS OF SIX REPLICATES FOR PERCENTAGE LOOSE SMUT, TOTAL NUMBER OF SPIKES, AND YIELD IN GRAMS OF SIX LEVELS OF INFECTION WITH *Ustilago nuda* ON NEWAL BARLEY GROWN AT CASTOR, ALTA., IN 1939

Treatment	Percentage loose smut	Total number of spikes*	Yield*, gm.
Infected seed	11.5	376	353
4 Parts infected/1 part non-infected	8.9	394	376
3 Parts infected/2 parts non-infected	6.1	416	380
2 Parts infected/3 parts non-infected	4.6	398	377
1 Part infected/4 parts non-infected	1.5	414	382
Non-infected seed	0.2	438	427

* *F* value obtained for 5 D.f. for treatment and 20 D.f. for error is 0.91 for total number of spikes and 1.9 for yield, both being non-significant.

No statistically significant regressions for yield on the percentage loose smut were demonstrable among the data obtained at Fallis and Castor. The lowest yield was found in the treatment showing the mean maximum of smut infection at both stations. This tendency for a relationship suggested the repetition of the trials in 1940.

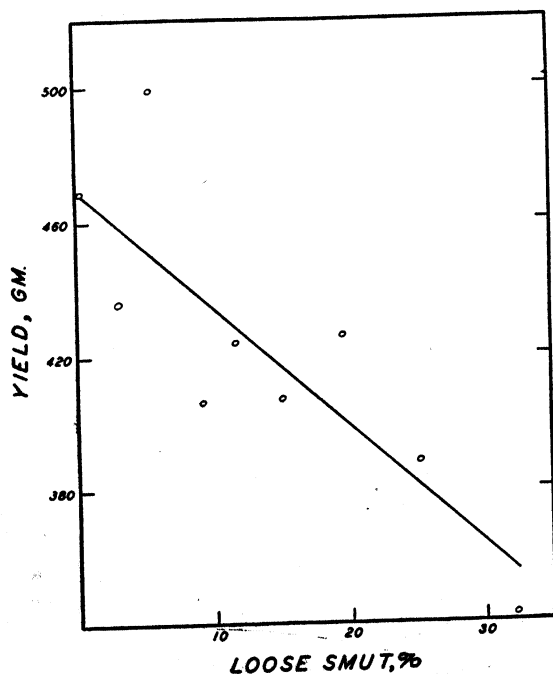


FIG. 1. Regression of yield on percentage loose smut in Newal barley grown at St. Paul, Minn., in 1940.

The 1940 Yield Trials

Yield trials in 1940 were conducted at three stations: St. Paul, Minn.; Edmonton and Fallis, Alta.

St. Paul, Minn.

The results obtained at St. Paul, with nine levels of loose smut, are presented in Table III and the regression of yield on percentage loose smut is graphically represented in Fig. 1. The maximum mean percentage of loose smut at this station was 32.5 and the minimum 0.3. Highly significant differences for treatment yields were obtained. The minimum yield of 342 gm. occurred with the treatment in the highest infection level, namely, 32.5% loose smut, and the maximum yield of 499 gm. occurred in the treatment showing 5.5% loose smut. The regression of yield per plot on percentage loose smut was highly significant (P less than 0.001) with a 1% increment of loose smut reducing yield 0.85% or 3.6 gm.

TABLE III

MEANS OF SIX REPLICATES FOR PERCENTAGE LOOSE SMUT, TOTAL NUMBER OF SPIKES, AND YIELD IN GRAMS OF NINE LEVELS OF INFECTION WITH *Ustilago nuda* ON NEWAL BARLEY GROWN AT ST. PAUL, MINN., IN 1940

Treatment	Percentage loose smut	Total number of spikes	Yield, gm.
Infected seed	32.5	514	342
8 Parts infected/2 parts non-infected	25.2	523	388
6 Parts infected/4 parts non-infected	19.5	508	426
5 Parts infected/5 parts non-infected	15.1	489	407
4 Parts infected/6 parts non-infected	11.6	489	424
3 Parts infected/7 parts non-infected	9.1	529	406
2 Parts infected/8 parts non-infected	5.5	494	499
1 Part infected/9 parts non-infected	3.1	486	436
Non-infected seed	0.3	488	469

Difference necessary for significance at the 5% point = 50 spikes, 58.8 gm.

Edmonton, Alta.

The data for Edmonton are presented in Table IV and the regression of yield on percentage loose smut is shown in Fig. 2. At this station only six percentage levels of infection were included in the yield test with the maximum amount of loose smut at 30.5% as a treatment mean and the minimum at 0.2%. The yield varied correspondingly from 280 gm. at the mean minimum to 427 gm. as the mean maximum. Highly significant differences in yield were obtained with the six percentage levels of loose smut. The regression of yield on percentage loose smut was highly significant (P less than 0.001) with a 1% increment of loose smut reducing yield 1.4% or 4.8 gm.

Fallis, Alta.

The data for this station are summarized in Table V and the regression of yield on percentage loose smut is indicated in Fig. 3. Six percentage levels of loose smut were included in this yield test as at Edmonton. Samples from

TABLE IV

MEANS OF SIX REPLICATES FOR PERCENTAGE LOOSE SMUT, TOTAL NUMBER OF SPIKES, AND THE YIELD IN GRAMS OF SIX LEVELS OF INFECTION WITH *Ustilago nuda* ON NEWAL BARLEY GROWN AT EDMONTON, ALTA., IN 1940

Treatment	Percentage loose smut	Total number of spikes	Yield, gm.
Infected seed	30.5	361	280
4 Parts infected/1 part non-infected	24.8	359	295
3 Parts infected/2 parts non-infected	16.5	370	341
2 Parts infected/3 parts non-infected	11.1	359	341
1 Part infected/4 parts non-infected	6.8	381	394
Non-infected seed	0.2	396	427

Difference necessary for significance at the 5% point = 28 spikes, 32.1 gm.

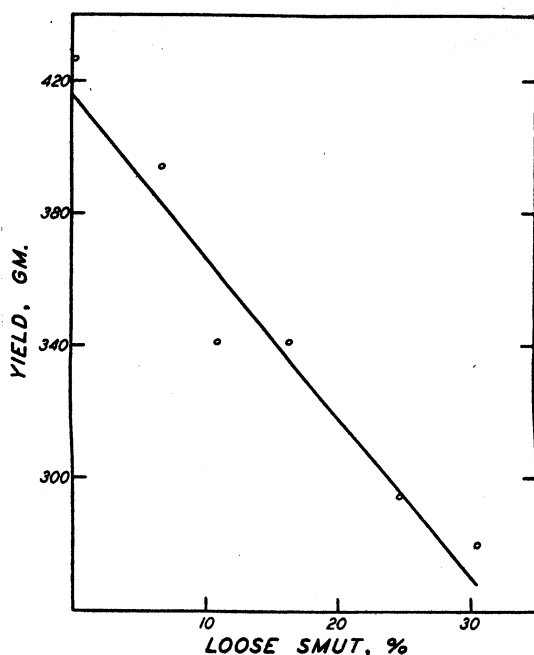


FIG. 2. Regression of yield on percentage loose smut in Newal barley grown at Edmonton, Alta., in 1940.

the same infected seed lots were used at both stations. At the Fallis station the maximum occurrence of loose smut was 32.4% as a treatment mean and the minimum 0.5%. The yield varied correspondingly from 141 to 207 gm. The variance in yield at this station was the lowest of the three stations in 1940. Highly significant differences in yield were obtained with the six different levels of loose smut. The regression of yield on percentage of the disease was highly significant (P less than 0.001). A 1% increment of loose smut reduced yield 1.2% or 2.1 gm.

TABLE V

MEANS OF SIX REPLICATES FOR PERCENTAGE LOOSE SMUT, TOTAL NUMBER OF SPIKES, AND YIELD IN GRAMS OF SIX LEVELS OF INFECTION WITH *Ustilago nuda* ON NEWAL BARLEY GROWN AT FALLIS, ALTA., IN 1940

Treatment	Percentage loose smut	Total number of spikes	Yield, gm.
Infected seed	32.4	297	141
4 Parts infected/1 part non-infected	24.8	315	156
3 Parts infected/2 parts non-infected	18.4	314	166
2 Parts infected/3 parts non-infected	13.0	315	178
1 Part infected/4 parts non-infected	5.5	304	198
Non-infected seed	0.5	322	207

Difference necessary for significance at the 5% point = 49 spikes, 11.0 gm.

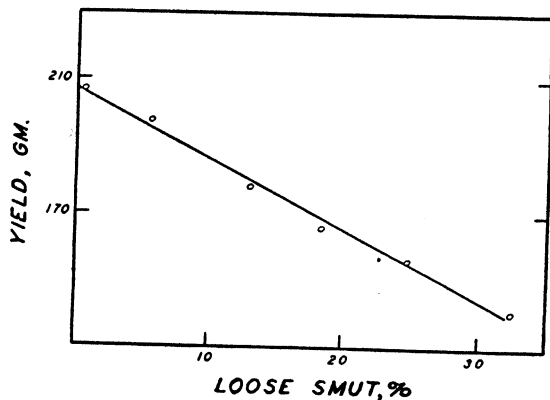


FIG. 3. Regression of yield on percentage loose smut in Newal barley grown at Fallis, Alta., in 1940.

Influence of Rate of Seeding of Infected Barley Seed on Yield

One difference in the loose smut yield test trials conducted in 1939 and in 1940 was in the rate of seeding. In 1939, 14 gm. of seed, approximately 370 seeds, were drilled in each 18 ft. row while in 1940, 400 seeds were planted in rows of the same length. A yield test was therefore conducted in St. Paul in 1940, in a randomized block with four replications; infected and non-infected seed was used to determine the influence that rate of seeding had on the incidence of loose smut and the consequent reductions in yield. The rates of seeding were: 108 seeds, 216 seeds, and 648 seeds per row. Each plot contained three rows 18 ft. long and one foot apart; only the centre row was harvested. The results obtained are summarized in Table VI. Significantly higher percentages of loose smut occurred at the higher rates of seeding. Seeds artificially infected with *U. nuda* showed 26.9, 30.1, and 34.0% loose smut, respectively, for seeding rates of 108, 216, and 648 seeds per plot. Non-infected seed showed 0.0, 1.2, and 0.8% loose smut, respectively, at

TABLE VI

RATE OF SEEDING AND INCIDENCE OF LOOSE SMUT ON YIELD OF NEWAL BARLEY AT ST. PAUL, MINN., 1940

Rate of seeding, No. seeds per row	Seed used	Percentage infection	Total number of spikes	Mean number of spikes per seed sown	Yield, gm.
108	Infected	26.9	334	4.0	329
	Non-infected	0.0	335	4.0	442
216	Infected	30.1	432	2.6	330
	Non-infected	1.2	414	2.5	480
648	Infected	34.0	587	1.2	355
	Non-infected	0.8	509	1.0	498

these same rates of seeding. Yield per plot from non-infected seed was significantly higher at all rates of seeding than the yields from the infected seed.

No statistically significant differences in yield were found between the three rates of seeding within either the infected or non-infected seed lots. The very similar yields obtained in this test suggest that small variations in the amount or number of seeds sown per row do not affect yield to any appreciable amount.

Incidence of Loose Smut Compared on the Basis of Total Number of Plants and of Total Number of Spikes

The method used in the present study of recording the incidence of loose smut on the basis of total number of infected and non-infected spikes was compared with the percentage of plants showing loose smut. For such a comparison, a side experiment was conducted at Edmonton in 1939 and 1940. Samples from the same seed mixture as used in the regular yield trials were obtained for the experiment to determine the percentage of smutted plants at the different levels of infection. Fifty seeds were spaced with a "V" belt seeder in an 8 ft. row in 1939 and 150 seeds were spaced in an 18 ft. row in 1940. This test was conducted in a quadruplicated randomized block arrangement. The percentage of plants showing loose smut was determined in early fall by pulling up the entire row of plants and recording the number of infected and non-infected plants. The results obtained are summarized in Table VII, together with the percentage of spikes showing loose smut as obtained from the regular yield trial.

In general, percentage values obtained for loose smut on the basis of number of plants infected were similar to those obtained on the basis of number of spikes infected. Close agreement was also obtained with the data from the other two stations at Fallis and Castor in 1939, and at Fallis and St. Paul. The fairly uniform results obtained in the incidence of loose smut at three widely different stations in 1939 and again in 1940 suggest that soil and climatic conditions have only a minor influence on the expression of this disease.

TABLE VII

PERCENTAGE LOOSE SMUT AS COMPARED ON THE PER PLANT AND THE PER SPIKE BASIS, AT COMPARABLE INFECTION LEVELS

1939				1940			
Spike basis			Plant basis	Spike basis			Plant basis
Fallis	Castor	Edmonton*	Edmonton	Fallis	Edmonton	St. Paul	Edmonton
10.8	11.5	8.7	9.0	32.4	30.5	32.5	35.8
9.4	8.9	6.6	4.2	24.8	25.8	25.2	25.4
5.8	6.1	4.9	4.2	18.4	16.5	19.5	20.6
4.0	4.6	4.5	3.7	—	—	15.1	—
2.3	1.5	2.3	3.4	13.0	11.1	11.6	12.4
0.1	0.2	0.0	0.0	—	—	9.1	—
				5.5	6.8	5.5	5.3
				—	—	3.1	—
				0.5	0.2	0.3	1.4

* Because of a damaging hail-storm, yield data were not obtained at this station.

Discussion

Loose smut of barley follows from a systemic infection of the barley plant by *U. nuda*. Although the influence of such an infection on tillering was not determined on the per plant basis in the present study, the results obtained in the regular yield trials indicated that tillering of plants, as determined by the number of spikes formed, was not affected in the early stages of seedling growth. The general agreement of values for percentage of infected plants with the percentage of infected spikes pointed to the occurrence of smut on all spikes produced by an infected plant. Support for this observation was found in the recent work of Oort (2), who noted that from 2290 plants infected with *U. nuda*, 96.2% showed all heads completely destroyed or, at most, not over one head of a plant less than one-half healthy. In only very few instances were partially diseased spikes encountered in the tests reported in this paper.

No satisfactory explanation can be offered for the lack of significant differences in yield between the different percentage levels of loose smut in 1939. Small variations in the number of seeds sown had little effect on the yield of grain when either infected or non-infected seed lots were used.

The differences between stations during 1940 in the percentage decreases in yield for every 1% increase in loose smut are not real. The regression coefficient of 0.85% at St. Paul is not significantly lower than the coefficients of 1.4% and 1.2% for Edmonton and Fallis, respectively.

Acknowledgments

The authors are indebted to Dr. K. W. Neatby, formerly Professor of Field Crops, University of Alberta, for suggesting this study and for his advice in the preparation of the manuscript. Dr. F. R. Immer, Professor of Agronomy

and Plant Genetics, University of Minnesota, willingly advised in the analysis and the interpretation of the data. His help is gratefully acknowledged. They wish to thank Dr. H. K. Hayes, Chief of the Division of Agronomy and Plant Genetics, University of Minnesota, for permission to carry on the test, and for field assistance, at the University Farm, St. Paul, Minn.

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HOST-PARASITE RELATIONSHIPS IN A SEED-BORNE DISEASE OF BARLEY CAUSED BY *HELMINTHOSPORIUM SATIVUM* PAMMEL, KING, AND BAKKE¹

By H. W. MEAD²

Abstract

A hulled barley kernel is a caryopsis enclosed by the tightly adhering floral glumes. The testa, which is formed from the inner integument of the ovule, becomes increasingly resistant to fungi. Spikelets may become parasitized during flowering and afterwards, by air-borne spores and fragments of mycelium of *H. sativum*. The fungus may cause blighting, shrivelling, and discoloration of the spikelets and maturing kernels, the damage depending upon the time of inoculation. Irregular, dark, thick-walled mycelium may be found massed in the parenchyma of the glumes, pericarp, and lodicules, and ungerminated spores between the glumes and pericarp. This dormant mycelium will remain viable for from two to five years. It germinates when the kernel germinates and infects the young tissues of the plumule and radicle as these organs expand and force their way through and past the infected tissues. It also colonizes the soil nearby, to a limited extent. Embryo blight, pre-emergence and postemergence blight, with malformation, stunting, and lesioning of the seedling may follow infection, the amount of each depending upon certain environmental factors.

Introduction

Many seed samples of barley received from various districts in Canada contain kernels that are infected with the fungus *Helminthosporium sativum* Pammel, King, and Bakke. This fungus may become established in the kernel while it is developing and it may infect various parts of the embryo during germination. The purpose of this study was to determine the relation of the fungus to various parts of the kernel and developing embryo.

The disease on the kernel usually is indicated by a diffuse pale to dark brown discoloration of the lemma and palea, especially in the region of the embryo and groove (Fig. 1, A, B). This staining may spread over the dorsal surface of the kernel or occur as isolated brown spots, or at times the fungus may be present without any symptoms being evident. Infected kernels may be shrivelled, but they usually are as plump or plumper than healthy kernels. The terms smudge, kernel smudge, and black point have been used to describe the affected kernels, but these terms have been used also to describe similar discolorations caused by other fungi. The term smudge is being used in this paper.

The disease on the seedling may take the following forms: embryo blight, pre-emergence and postemergence blight, with malformation, stunting, and lesioning of the seedling. The plumule and roots may be rotted before they emerge from the hulls of the kernel, or later they may become distorted and

¹ Manuscript received in original form January 26, 1942, and as revised, July 10, 1942. Contribution No. 712 from the Division of Botany and Plant Pathology, Science Service, Dominion Department of Agriculture, Ottawa, Canada. Based on part of a thesis submitted to the University of Toronto in 1942 in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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weak, and unable to penetrate the soil. The majority of the seedlings emerge from the soil. Of these, a few may remain in a stunted condition for a time and then die. The rest may develop in an apparently normal manner but most of them bear brown, circular to oblong lesions on the roots and coleoptile and occasionally on the leaves.

This characteristic discoloration of barley kernels has been observed and described in Europe and North America. Zöbl (23) called it "Braunspitzige", and concluded that *Cladosporium herbarum* (Pers.) Lk. caused the discoloration. Puchner (15) called it "Schwartzspitzige" and found that discoloured kernels germinated poorly and after germination often produced seedlings with lesions on the leaves. Ravn (17) also used the term "Braunspitzige", but attributed it to species of *Helminthosporium* of which *H. teres* Sacc. was found to occur frequently in cultures of discoloured barley kernels. Peyronel's (14) "Puntatura" appears to refer to the same malady. He said that the integuments of the kernel became discoloured owing to the presence of a brown pigment, and that although the pigment was confined chiefly to the layer of horizontal cells overlying the epicarp, it sometimes occurred in the epicarp as well. The cells of the aleurone layer often were found to be discoloured but not those of the embryo. The first symptom of the disease was a discoloration of the lodicules which are close to the scutellum. At the base of these there was found a thin-walled tissue rich in nutrients, which collapsed early. This was considered to be a likely substratum from which the fungus might invade the integuments overlying the embryo. He showed that *C. herbarum* was sometimes associated with the discoloration, but not the cause of it. Rosella (18) found that *Helminthosporium gramineum* (Rab.) Erik. caused a seed discoloration in barley which he called "Moucheture". He observed also that *H. sativum* invaded barley spikes while they were still enclosed by the leaf sheaths. The fungus penetrated the sheaths and young glumes and invaded the kernels. Christensen and Stakman (2) described "seed blight" in barley in which heavily infected seeds were discoloured, shrivelled, and of inferior quality and from which seedling blight, root rot, basal stem rot, and decrease in yield often resulted. They found species of *Fusarium*, *Helminthosporium*, and *Alternaria* to be the fungi most commonly present. Güssow (9) reported a seed-borne disease of barley that was caused by *H. sativum*. It caused spotting and shrivelling of the grain and leaves. Machacek and Greaney (12) described a similar discoloration in the seeds of cereals, which was referred to as black point or kernel smudge. They considered that it was caused mostly by fungi, the predominating forms being *Alternaria* spp., *H. sativum*, *H. teres*, and *Fusarium* spp. In certain years, one of these fungi was much more common than the others on cereal seeds. For instance, in 1935, *H. sativum* was predominant, and in the period 1931 to 1934, two species of *Alternaria* were predominant. Dickson (5) described the condition under the heading, spot blotch of barley, *Helminthosporium sativum* P.K. & B. The discoloured and sometimes shrunken kernels were described as "blight damaged" and it was pointed out that they are given the

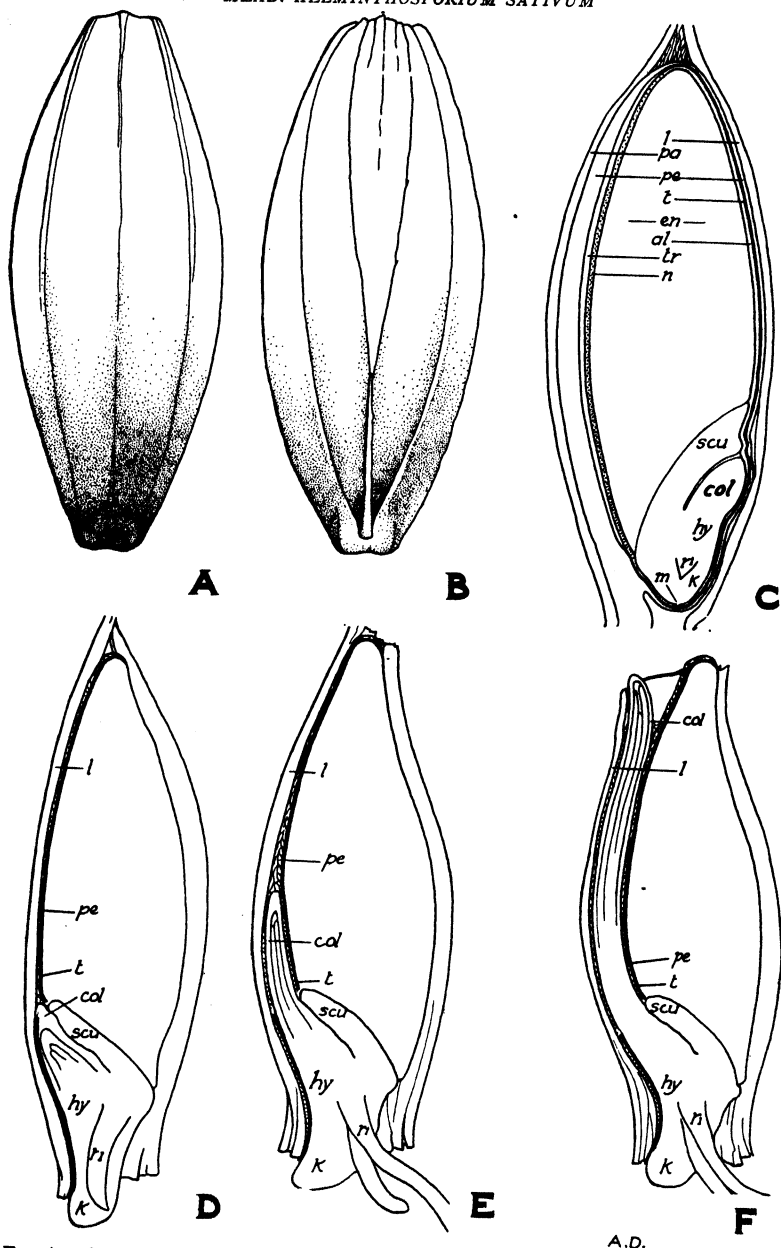


FIG. 1. Surface views and longitudinal sections of hulled barley kernels. A, B, dorsal and ventral surfaces respectively of smudge kernels, showing by means of shading, the location of the typical discoloration; C, median longitudinal section showing the extent and relation of the parts of a dormant kernel; D, E, F, longitudinal sections showing the relation of the parts of germinating kernels, 2.5, 3.5, and 5.5 days, respectively, after being placed in germinators at 10° C. Al, aleurone; col, coleoptile; en, endosperm; hy, hypocotyl; k, coleorhiza; l, lemma; m, micropyle; n, nucellus; pa, palea; pe, pericarp; r, primary root; scu, scutellum; t, testa; tr, transchalazal tissue. $\times 9$ approx.

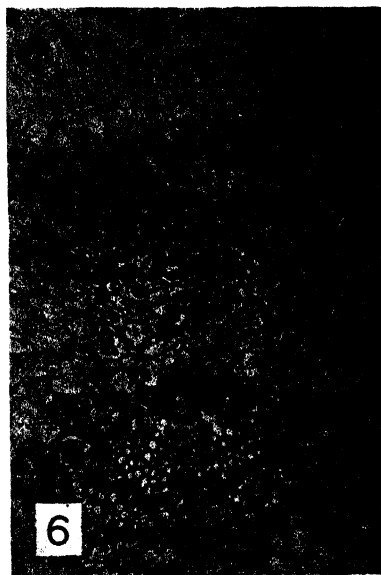
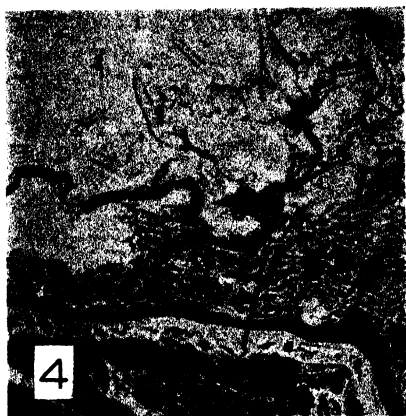
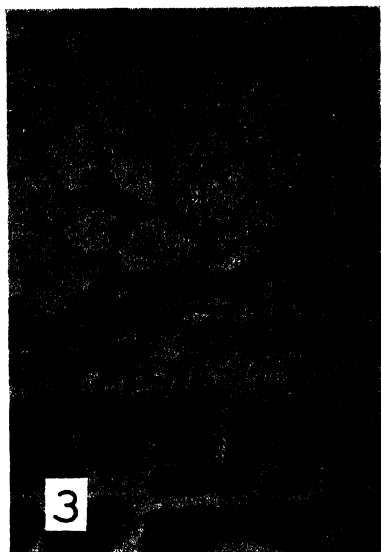
special grade designation "blighted" in the Federal Grain grades of United States. The disease is common in the southern part of the barley zone but is considered to be of minor importance. In Nebraska, in 1941, this disease was common, many samples of barley containing as high as 38% blighted kernels (8). In Canada, the most severely infected samples have been obtained from New Brunswick and Manitoba.

The Development and Structure of a Healthy Hulled Barley Kernel

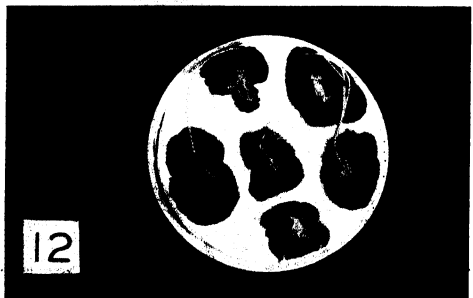
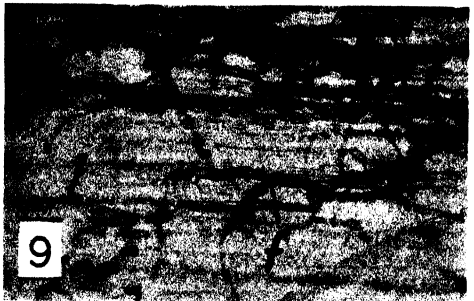
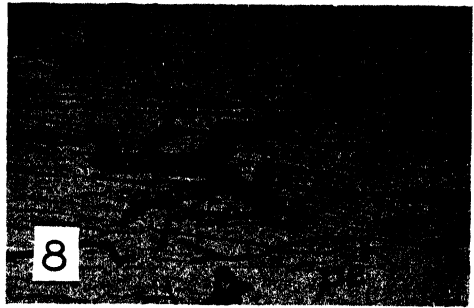
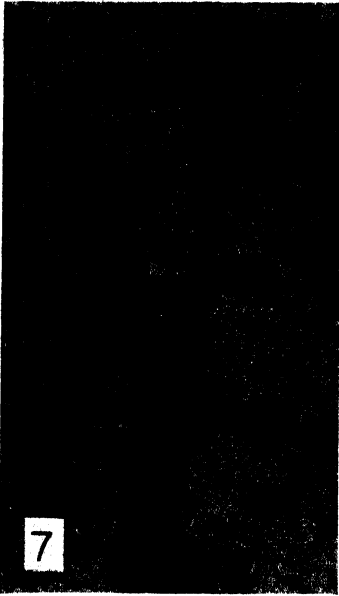
A hulled barley kernel is a caryopsis closely invested by the floral glumes (Fig. 1C, *l, pa*). These glumes, lemma and palea, begin to adhere to the pericarp (ovary wall) about 20 and 10 days, respectively, after flowering (10). They consist of thick-walled ground tissue, vascular strands, and longitudinal green bands of thin-walled chlorophyllous parenchyma. The outer epidermis consists of alternating large and small cells with thick walls. The walls have characteristic wavy thickening and simple pits. The cells of the inner epidermis have thinner walls. There are stomata on both inner and outer surfaces. Both epidermises become cutinized and silicated and when mature are very hard in texture. The outer glume, the lemma, overlaps the palea, and together they provide a hard protective covering for the caryopsis. When young, they are soft in texture and may become infected with fungous mycelium which also may grow over the edges and ends to reach the developing ovary. Spores of fungi also may lodge inside the glumes before the glumes begin to adhere to the pericarp.

The pericarp (the ovary wall) of the young caryopsis consists of several layers of parenchyma cells, arranged in two main layers. The inner, cross layer, is chlorophyllous and its cells are thick-walled (Figs. 2 and 3, *c*). There are inner and outer epidermal layers and these are thinly cutinized even at flowering time. They bear stomata and hairs. By the end of the first week after flowering, the inner epidermis has been absorbed and the pericarp itself is being absorbed (21) (Fig. 2, *pe*). Absorption of the pericarp is complete by the end of the second week and there is left a much flattened outer epidermis with a cuticle, covering a few rows of collapsed parenchymatous cells. This cuticle adheres to that of the glumes. The pericarp encloses the caryopsis completely (Fig. 1C, *pe*). It is thin over the embryo; in the region of the furrow it is thick and contains a vascular trace which extends along the length of the caryopsis (Figs. 5 and 6, *v*).

The inner integument of the ovule (Figs. 3 and 6, *i*), consisting of two layers of cells, eventually forms the testa of the mature caryopsis (Figs. 2, 4, and 5, *t*). As a result of compression and absorption it forms a narrow homogeneous, brown, oily-appearing layer bordered on both sides by cutin membranes, the outer being much thicker than the inner. This outer membrane increases in thickness from flowering to maturity. The testa completely envelops the caryopsis (Fig. 1C, *t*) except over the micropyle and in the



FIGS. 2 TO 6. Cross and longitudinal sections of normal barley kernels, stained with erythrosin and malachite green. *Al* = aleurone; *c* = chlorophyllous (cross) layer of pericarp; *en* = endosperm; *ep* = epidermis of pericarp; *ep.n* = epidermis of nucellus; *i* = inner integument; *o.cu* = outer cuticle of inner integument; *pe* = pericarp; *s.c(n)* = sheaf cells (nucellus); *t* = testa; *tr* = transchalazal tissue; *v* = vascular trace of pericarp. FIG. 2. Cross section of dorsal surface of kernel, almost mature. $\times 375$. FIG. 3. Dorsal surface of kernel seven days after flowering. $\times 375$. FIG. 4. Longitudinal section of apex of kernel, almost mature. $\times 180$. FIG. 5. Region of furrow almost mature. $\times 200$. FIG. 6. Cross section of region of furrow, seven days after flowering. $\times 200$.



FIGS. 7 AND 8. Strips of the pericarp of the kernels naturally infected with *H. sativum* showing dormant mycelium of the fungus (f). $\times 350$. FIG. 9. Mycelium in the lemma. $\times 200$. Stained with acid fuchsin. FIG. 10. A longitudinal section showing mycelium (f) of *H. sativum* in the embryo sac of a spikelet inoculated at flowering time. $\times 200$. Stained with erythrosin and malachite green. FIG. 11. *H. sativum* on the surface of a naturally infected kernel after five days in a moist chamber at 24°C . $\times 15$. FIG. 12. Colonies of *H. sativum* from infected kernels on potato dextrose agar for seven days. $\times 1/3$.

region of the groove where it originates sharply at either side of the vascular trace (Figs. 5 and 6). Between the flanks of the testa is a strand of chalazal cells, the base of the ovule (Figs. 5 and 6, *tr*). The chalazal cells become thick-walled, suberized, and filled with fatty deposit (21). This is evident by the 12th day after flowering. It has been suggested by Krauss (11) that in some varieties of barley the outer cuticle of the integument and at times the inner cuticle may be absent from the micropylar area. Tharp (21) found them to be much thinner in that region than elsewhere. These cutin membranes are thick over the apex of the caryopsis (Fig. 4, *t*) and in the flanks of the furrow. The entire testa (integument plus membranes) varies in thickness. It is thin over the dorsal and lateral surfaces, distinctly thin over the embryo, thick over the apex of the kernel and at the flanks of the furrow.

The nucellus of the ovule becomes absorbed by the developing endosperm and is represented in the mature kernel by a structureless layer inside the testa over the dorsal surface and flanks, and a group of glandular cells in the region of the furrow (Figs. 5 and 6, *s.c(n)*). This group of cells, called "sheaf cells" by Tharp (21) and Collins (3) is thought to be part of the nucellus and serves to distribute supplies to the endosperm and embryo from the vascular trace of the pericarp. In wheat it is considered to be a barrier to fungous invasion because of its thickness and chemical composition (16).

The aleurone layer is a complete envelope except at the micropyle, where it is discontinuous (Fig. 1C, *al*). It consists of a single layer of elongate cells over the embryo and three or four layers of rectangular cells over the face and flanks of the kernel (Figs. 2 and 3, *al*). In the region of the furrow it has an irregular structure. At maturity this layer contains storage protein.

The endosperm develops by absorption of the nucellus. At maturity its contents are relatively dry and consist of hexose compounds. This condition is thought to be a barrier to extensive fungus invasion of the endosperm of wheat (16).

The embryo consists of a short hypocotyl, at the apex of which is the plumule enclosed by the coleoptile (Fig. 1C, *col*) and at the base the radicle consisting of the root initials enclosed by the coleorhiza (Fig. 1C, *k*). Attached to one side of the axis and forming the greater portion of the embryo is the scutellum (Fig. 1C, *scu*). Percival (13) found that there were small globules of a fatty oil and granules of protein in the parenchyma of the scutellum and other parts of the embryo of wheat. Soon after germination starts, starch appears in the coleorhiza, root cap, and tip of the coleoptile, derived from fat and a soluble carbohydrate stored in the tissues of the embryo.

From the preceding description, it appears that the barley spikelet would be most susceptible to fungus invasion at flowering time and during the week following that period. During this time its defences are rather weak and some of its tissues provide a good substratum for the fungus. The cutin membranes of the pericarp and testa are present at flowering time but are thin. The glumes are soft at first and have not become sealed to the pericarp and the lodicules are distended for a time with nutrient materials. These young

tissues are readily parasitized by *H. sativum* and they provide nourishment for the fungus. There are certain structural weaknesses at this time. The areas around the micropyle and over the embryo are thinly covered and the chalazal tissue at the flanks of the testa has not developed its suberin lining. However, these weaknesses soon disappear and the kernel becomes enclosed in a resistant envelope, reinforced by hard glumes which adhere tightly. Similarly, the nutrient substances available to the fungus in the early stages disappear and the tissues which supplied them, collapse. These changes place a limit on the period during which air-borne fungi can successfully infect the kernel.

Chemical Changes during Development of the Spike

The writer has demonstrated the presence of reducing sugars in the parenchyma of the palea and lemma of young barley kernels two to three days after flowering, and again at 10 and 12 days after flowering. There were reducing sugars present also in the chlorophyll layer of the pericarp of 10-day old kernels. Tharp (21) reports a gradual change in the nature of the cell walls of the inner integument after the fifth day. The soft cellulose becomes less easily hydrated and thickens. At the same time the outer cutin membrane increases in thickness. At this time there are disorganized masses of endosperm and nucellar tissue in the regions of the embryo and chalaza. These are composed partly of much-hydrated cellulose and give a positive test for mucilage. The cells of the endosperm are filling with starch, and protein grains form in the aleurone during the second week after pollination; fat deposits are present in the mature kernel but at that time are very resistant to fat solvents. Eckerson (7) studied the chemical development of wheat from the primordia of the flower to the mature grain. Her findings with respect to the period following heading, i.e., the period during which the spike is exposed to wind-borne spores, are as follows. The pericarp breaks down rapidly and its contents, amino acids, glucose, fructose, and starch, move into the developing embryo sac to the embryo via the chalazal tract and nucellus. This movement is rapid at the time wall formation in the endosperm is beginning and continues during the development of the kernel. The nucellus soon breaks down, with only the outer membrane remaining. At the time morphological development of the endosperm is complete, a stream of sugar and amino acids is passing from the rachis through the chalaza and endosperm to the embryo. Starch begins to form in the endosperm soon after wall formation and it continues to increase in amount until desiccation begins. There are amino acids and a small amount of fat in the endosperm also. The aleurone layer at this time contains magnesium, phosphate, and calcium with storage protein appearing later. The latter, with gluten, appears in the endosperm only at the time desiccation begins. At flowering time, the stigma contains much pectin and a little glucose. These substances are exuded and also are present all the way to the embryo sac.

Histological Studies of Barley Kernels Infected with *H. sativum*

A. Materials and Methods

A diseased sample of Charlottetown No. 80 barley from New Brunswick, and a sample of Colsess barley from Manitoba were used in this study. The disease on the seed was studied by means of stained strips of the seed coverings and by microtome sections. Thin strips of glumes, pericarp, and root and shoot tissue were boiled in lactophenol containing cotton blue, or acid fuchsin and were mounted in lactophenol. Diseased mature kernels were soaked in water, the hard palea and lemma removed, and the remainder fixed in formalin acetic alcohol (1). Kernels in various stages of development, inoculated with the fungus *H. sativum*, also were fixed. Butyl alcohol was used for dehydration. Serial sections 10 to 15 μ in thickness were stained with a saturated aqueous solution of erythrosin and a weak solution of malachite green in absolute alcohol. The early stages of seedling infection during germination were studied by means of strips and microtome sections also. Solubility and staining tests for membrane substances were applied to hand sections of the coleoptile as follows: pectin, soluble in hot water; pectic acid, soluble in warm two per cent potassium hydroxide or ammonia; calcium pectate, soluble in hot potassium hydroxide after heating in two per cent hydrochloric acid; cutin, stains yellow or brown in chloro-iodide of zinc.

B. Examination of Field Material

1. Development in Culture

In order to establish the presence of *H. sativum* in the two seed samples of barley that were thought to be infected with the fungus, representative lots were plated on potato dextrose agar and on moist filter paper (Figs. 12 and 11). The New Brunswick sample contained many kernels of the smudge type and the rest of the kernels had a weathered appearance. Some of this seed was surface sterilized by immersing it for three minutes in a mixture containing one part of 95% alcohol and three parts of 1 : 1000 aqueous solution of mercuric chloride, and then rinsing it in sterile water. Kernels taken at random from the surface sterilized lot, were plated on moist filter paper and incubated at 24.5° C. for four days. *H. sativum* developed on all of the kernels. The Manitoba sample was tested four times on potato dextrose agar and on moist filter paper. *H. sativum* grew from over 95% of the kernels in each test.

2. Occurrence of Mycelium in the Coverings of Naturally Infected Kernels

An examination of the tissues of kernels from these samples was undertaken to determine the location of the fungus. For this purpose the mature kernels were soaked in formalin acetic alcohol overnight and then strips of the lemma, palea, and pericarp were removed. These strips were stained and examined with a microscope. The results are given in Table I.

TABLE I

Helminthosporium sativum IN TISSUES OF MATURE BARLEY KERNELS

Tissue	Occurrence of mycelium in strips from:								
	Proximal end		Centre		Distal end		Both ends	Entire surface	Total
	A	B	A	B	A	B	A	A	A
	%		%		%		%	%	%
Lemma	12	++++	3	+	21	+++	0	24	60
Palea	26	++++	8	++	26	+++	3	13	76
Dorsal pericarp									
Outer layer	17	++++	8	+++	13	+++	13	4	55
Inner layer	20	+	10	++	11	++	12	6	59
Ventral pericarp									
Outer layer	11	+++	2	+	4	+++	0	11	28
Inner layer	0	0	0	0	0	0	0	0	0

NOTE: A = Strips containing mycelium.

B = Prevalence of mycelium.

The results show that mycelium was present to some extent in all of the seed coverings except in the innermost layer of the pericarp on the ventral side of the kernels. It was not found in every strip, nor was it co-extensive with them. It was present more commonly towards the ends of the kernels, particularly the proximal end, where most of the discoloration occurs. The depth of penetration by the fungus is indicated by the presence of hyphae in the inner layer of the pericarp on the face of the kernel. Hand sections failed to show deeper penetration. No mycelium was found in the embryo of mature kernels. It will be shown later that infection of the embryo caused blighting and shrivelling and such kernels are blown away during cleaning.

The mycelium on the surface of the cells was, for the most part, dark, thick-walled, and highly septate, and it contained protoplasm. Acid fuchsin stained the protoplasm deeply and left the walls very lightly stained. The strands appeared in various forms, some being short and grouped to form plates, or ladder-like formations (22), others being long and branched. Single strands containing only a few cells were found also (Figs. 7 and 8, f).

This mycelium was found to be on the surface, between and within the cells of the various seed coverings. There was a tendency for the strands of mycelium on the outer pericarp to be almost entirely on the surface of its outer epidermis, although some penetration was evident. The mycelium in the glumes had penetrated the thin walls of the parenchyma and could be found in the cells of the inner epidermis (Fig. 9). In the inner or cross layer of the pericarp, the hyphae were found to be both inter- and intracellular.

Points of penetration of the outer epidermis of the pericarp were observed. These points were indicated in the tissue when it was stained with acid fuchsin, by red areas on the otherwise lightly stained walls. In the centre of such an area there appeared a small translucent spot. This was the actual point of penetration. The remainder of the area, in which the colour became more

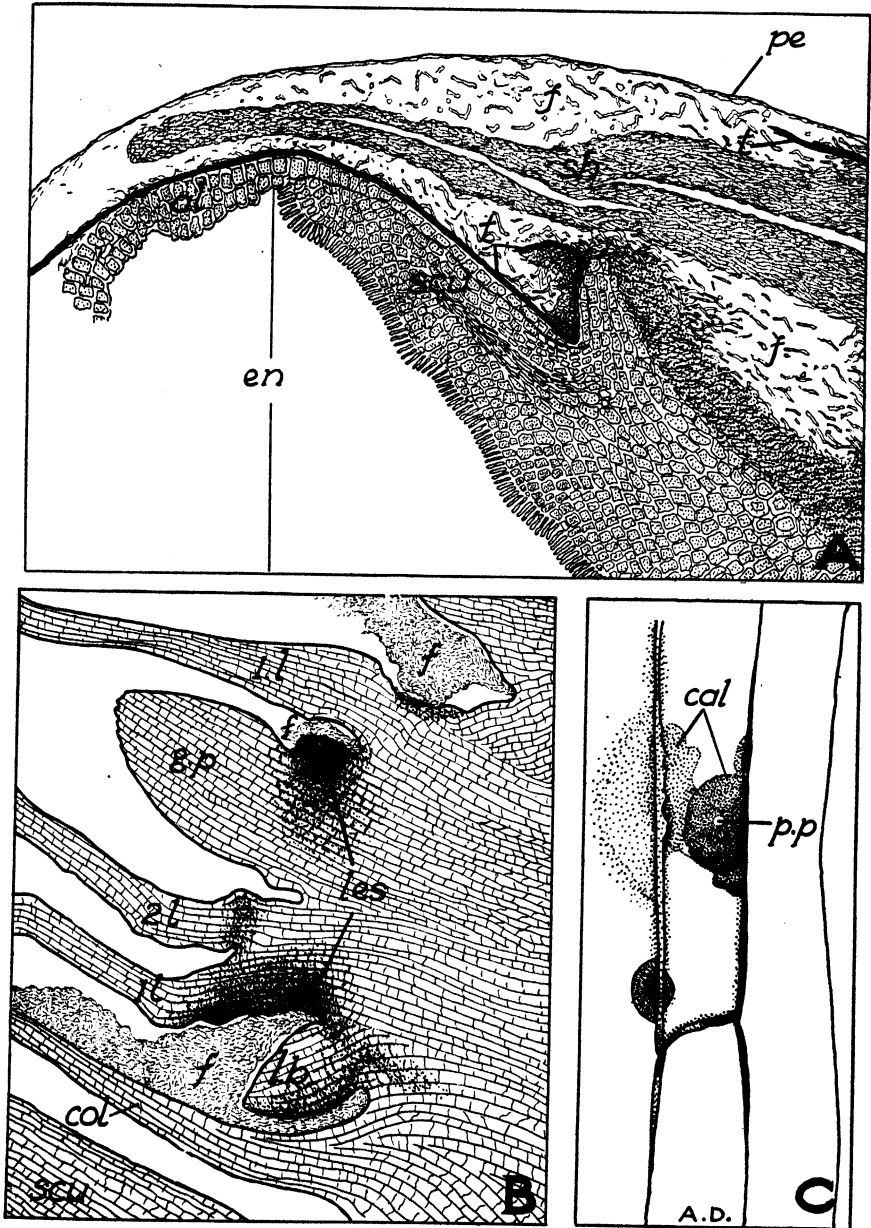


FIG. 13. Infection of barley seedlings by a seed-borne fungus, *H. sativum*. A, a longitudinal section of the upper portion of a blighted plumule (sh), showing the testa (t) ruptured, permitting entry of the fungus (f) from the pericarp (pe). The aleurone (al), scutellum (scu) and endosperm (en) were not infected, $\times 170$ approx.; B, a longitudinal section of the region of the growing point, showing lesions (les) at the base of the growing point (g.p.) first leaf (1l) and lateral bud (l.b.). The fungus (f) is massed in the axils of these organs, $\times 185$ approx.; C, an infected coleoptile cell showing callosities (cal) and thickening of the cell walls in the region of the penetration point (p.p.) and on a side wall, $\times 1700$ approx.

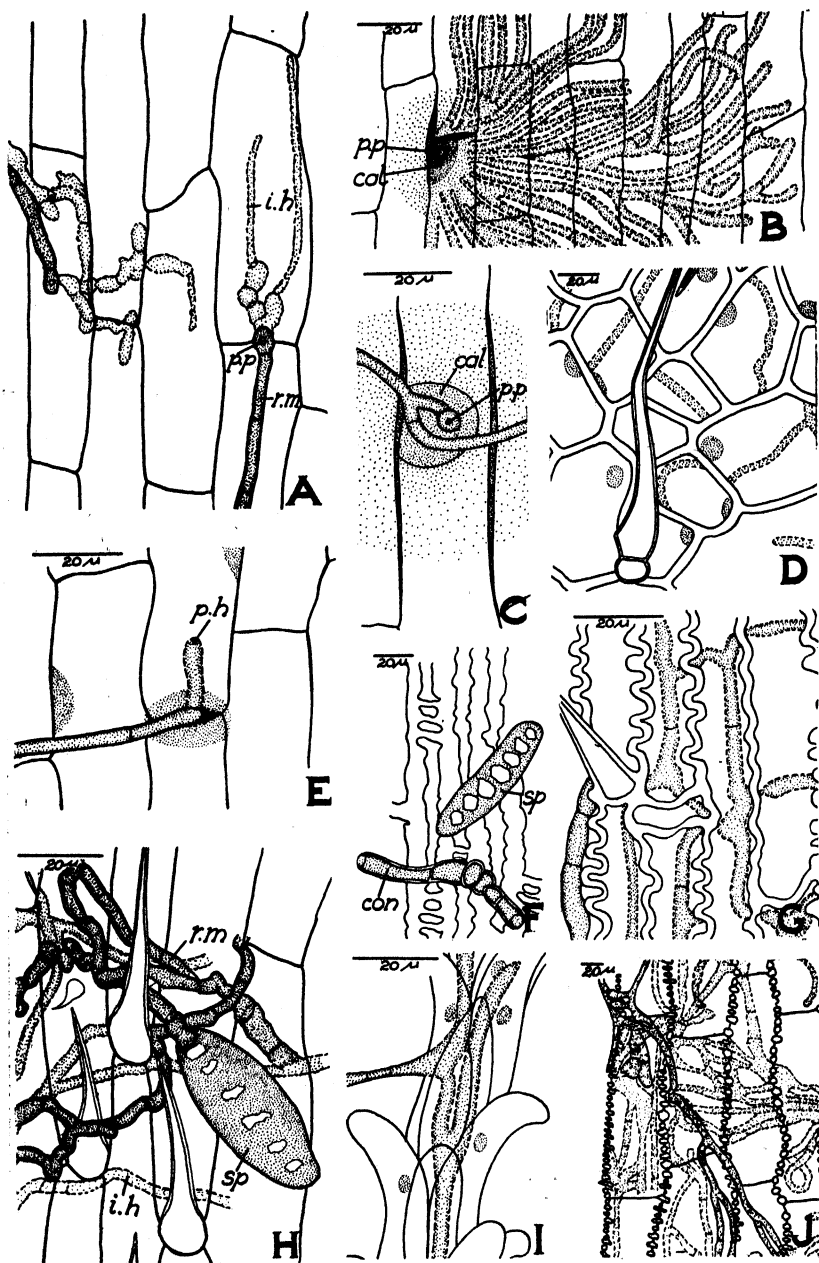


FIG. 14. *H. sativum* in tissues of barley kernels. A, resting mycelium (r.m.) on the surface of pericarp tissue, producing infection hyphae (i.h.) within the cells, 24 hr. after diseased kernels were moistened; B, mycelium spreading in the pericarp from a penetration point (p.p.); a callosity (cal) surrounds the penetration point; C, infection of a cell of the coleoptile during germination; D, mycelium in cells of the lodicules; E, penetration of a pericarp cell at a cross wall, showing the penetration hypha (p.h.) in the cell; F, conidiophore (con) of *H. sativum* growing from an opening in the outer epidermis of the lemma; a spore (sp) lying nearby; G, mycelium in the inner epidermis of the lemma; H, a spore (sp) of *H. sativum* producing mycelium on the surface of pericarp tissue and within the cells; I, mycelium in the stigma; J, dormant mycelium on the surface and within the outer epidermal cells of the pericarp.

and more diffuse toward the margin, represented what Dufrenoy (6) called a sheath which envelops the penetration point. Others (20, 22) called these structures calluses or callosities (Fig. 13C, *cal*). Invasion of the cells did not appear to cause their death immediately. Several cells were observed which contained hyphae but looked like healthy cells in other respects. For the most part, however, the invaded host cells had collapsed and contained a brown deposit. There was some indication of cell reaction in advance of the hyphae also. This was in the form of a slight yellowing of the cell walls and contents.

Mycelium was found in the remnants of the lodicules also (Fig. 14D). These structures, according to Peyronel (14) contain abundant nutrient materials at flowering time and might serve as a favourable substratum for a fungus. He considered that the rapidity of desiccation of the lodicules would affect the amount of disease in barley seed in different seasons.

3. Occurrence of Spores between the Glumes

Spores of *H. sativum* were found between the lemma and palea where the glumes overlap. In many instances, these spores had germinated and produced mycelium which grew inwards over the pericarp and outwards over the palea (Fig. 14H, *sp*).

C. Examination of Artificially Inoculated Kernels

Two methods of inoculation were used. In one, heads of barley grown in the greenhouse were dusted with finely ground oat hull inoculum of *H. sativum*. Some of these were incubated immediately in a humid atmosphere, others were left dry for various periods before incubating them. The other method involved spraying heads with a spore suspension and then incubating them in a moist chamber. The tissues were examined by taking strips, and making hand and microtome sections.

The following observations were made:

1. Heads Inoculated during Flowering or One to Two Days Later

Heads were inoculated, incubated for 48 hr., and then were examined. The fungus grew profusely over the surface of the glumes and sporulated. Strands of mycelium grew under the edge of the lemma and sporulated in the space between the lemma and palea. Penetration of the glumes was observed. The branches of the feathery stigma were infected early but the cells remained alive for a few days (Fig. 14I). The ovaries soon collapsed and became brown. Mycelium was observed in the embryo sac (Fig. 10, *f*) and in the lodicules (Fig. 14D). When dry inoculum was used, and the heads were left dry for seven days and then were incubated, it was found that fertilization had taken place and that the ovary had developed more or less normally but bore numerous lesions. The cells of the outer epidermis of the pericarp in the lesioned areas were packed with mycelium (Fig. 14J) the strands of which appeared to mass along the cell walls and break through as separate hyphae. These strands were irregular in diameter, and septa were distinguished with

difficulty. Hyphae were found among the cells of the inner layer of the pericarp. These hyphae also were very irregular in outline. Mycelium was seen in the outer epidermis of the lemma. The hyphae were reduced in diameter as they passed from cell to cell (Fig. 14G). Spikelets inoculated and incubated at flowering time were killed by the fungus.

2. Heads Inoculated 7 to 10 Days after Flowering

Mycelium was present throughout the tissues of the glumes and over their surfaces but continuity of the strands was not established. This surface mycelium passed between the edges of the glumes and spread over the pericarp. Points of penetration were observed, and the mycelium spread through the pericarp tissues (Fig. 14B). Freshly invaded cells were seen which had not collapsed, but there were also numerous lesions involving discoloured collapsed tissue. Most of the points of infection were over vertical walls (Fig. 14E) and it was evident, from the staining reaction, that there had been a chemical change in the cell wall for a considerable distance from the point of penetration. Microtome sections showed that the lesions in the pericarp were of considerable depth but that the fungus had not penetrated the inner integument (Figs. 17 and 18). The embryo, endosperm, and aleurone were free of mycelium. The kernels that developed were somewhat shrunken and discoloured.

3. Heads Inoculated 15 to 20 Days after Flowering

At this time, the glumes were adhering to the pericarp and were losing their green colour. The tissues of the glumes appeared to be fairly free of hyphae. There were hyphae in and on the surface of the pericarp, near both ends of the kernels, with very little on the central portions of the ventral and dorsal surfaces. This indicates that the ripening glumes acted as a barrier to the mycelium except at the ends where they are not completely closed. Penetration by the hyphae apparently had not gone beyond the pericarp. No hyphae could be found in the embryo, aleurone, and endosperm. The kernels that developed were plump, but were slightly discoloured.

These studies indicate that if inoculation of hulled barley kernels by a virulent form of *H. sativum* occurs at or soon after flowering, the ovaries are killed, and the other parts of the spikelet become infected. If inoculation occurs from 5 to 10 days later, the glumes, pericarp, and lodicules are invaded and areas of brown dead tissue occur in these organs. These areas contain living mycelium which is thick-walled and highly septate. Penetration of hyphae beyond the inner integument was not observed. Usually there was some shrivelling of the kernel at this stage, possibly as a result of the early collapse of infected pericarp tissues and consequent loss of food materials for the embryo and endosperm. It was observed frequently that filling of heads inoculated at that period was not complete. Some infection of 15- to 20-day-old kernels occurred, but it was confined mostly to the pericarp and to the parenchyma of the glumes at both ends of the kernel. There was no shrivelling at this stage.

Studies of Diseased Barley Kernels during Germination

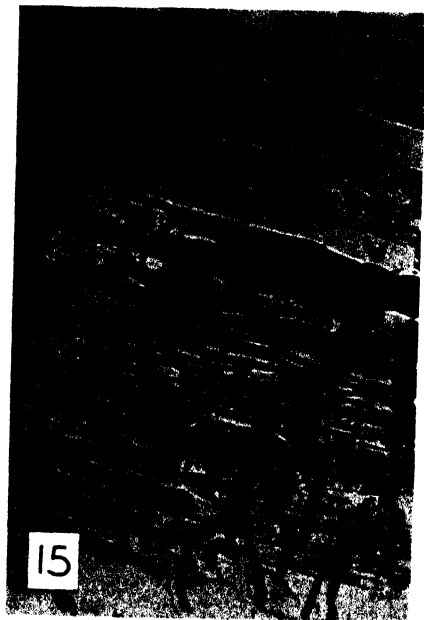
The following observations were made on diseased barley kernels and seedlings grown on moist blotters and in soil.

When the dry mature kernels come in contact with moisture they absorb it through their coverings. The pericarp and testa are thinnest over the embryo, and that is where the most rapid absorption occurs. When sufficient moisture has been absorbed (about 30% of the weight of the kernel in wheat (13)), and if oxygen is available and the temperature favourable, growth of the embryo begins. The first rupture of the testa and pericarp is over the expanding coleorhiza which protrudes and is in turn ruptured by the primary and lateral roots. The coleorhiza in its distended condition consists of thin-walled, elongate parenchymatous cells between which are many intercellular spaces. The coleoptile, through growth, expands towards the apex of the kernel. It ruptures the testa and aleurone at the upper end of the scutellum and forces its way longitudinally within the tissues of the pericarp (Fig. 1, D, E, F; Fig. 13A).

The same conditions that cause the kernel to germinate, cause the dormant mycelium and spores carried in the various tissues to start growing. It can be recalled that the tissues and organs that are in close contact with the growing embryo, viz. pericarp, glumes, and lodicules, were found to contain mycelium. The development of this mycelium in germinating kernels was studied by examining the surface of the kernels, strips of the pericarp, coleorhiza, roots, and coleoptile. The withered lodicules and the base of the shoot were examined also. Strips of the outer epidermis of the pericarp over the dorsal surface of kernels that have been incubated on moist filter paper in Petri dishes for 24 hr. contained both thick-walled resting mycelium and thin-walled new hyphae (Fig. 14A, *r.m., i.h.*). The thick-walled mycelium was on the surface of the tissue and swollen ends or short side branches were seen overlying penetration points. From these points, new thin-walled hyphae were seen inside the cells. They grew mostly lengthwise of the cells, but they were crossing them also, in their passage to neighbouring cells. In some cases, there was massing or swelling of the hyphae before they broke through the cell walls. The invaded cells were not killed immediately, but later they became necrotic, with brown contents and walls. When pericarp tissue of kernels, germinated at 24° C. for five days, was examined, it was found to contain hyphae throughout its thickness. The coleoptile enclosing leaf primordia and the growing point, forces its way through such infected tissue (Fig. 1, D, E, F) and is exposed to living hyphae during the passage (Fig. 13A, *f.*). The duration of this exposure depends upon the soil environment. Similarly, the coleorhiza and roots are in contact with the frayed edges of the pericarp which they rupture. Observations made on this torn pericarp showed hyphae growing from its edges and in contact with the coleorhiza where penetration points were found. Other hyphae grew from the forward edge of the coleorhiza across a considerable space to the surface

of the primary root where they ended in short branches. Strips from the surface of the root contained penetration points. Similarly, hyphae grew from beneath the lemma at the distal end of the kernel, and spread over the coleoptile, where penetration occurred (Fig. 14C, *p.p.*). Other internal sources of inoculum are the lodicules. Located as they are at the base of the kernel, they serve as a point of attack upon the coleorrhiza and roots. In germinating kernels these bodies were found very frequently covered with conidiophores and conidia of *H. sativum*. The roots and shoot may be attacked from the surface inoculum also. As already described, the fungus sporulates freely on the surface of diseased kernels (Fig. 11). Some of this mycelium comes from beneath the edge of the lemma, and some through the lemma (Fig. 14F, *con*). Direct examination of this surface growth showed that strands of the mycelium were stretched across the space between the ends of the glumes and the surface of the coleoptile, coleorrhiza, or roots. Detached conidia were found floating in films of water which had formed along the kernel and coleoptile, and penetration points on the coleoptile were found at this location.

A careful examination of the hypocotyl region of the embryo (Fig. 1, D, E, F, *hy*) of germinating kernels was made. In some cases, dark lesions of considerable depth were present on the dorsal surface of this region (Fig. 19, A, B), and when portions of the discoloured tissue were crushed and examined, mycelium was found in the external layers. Considerable discoloration was seen on the dorsal surface of the distal portion of the scutellum, where it is in contact with the base of the coleoptile. On the surface of this depression strands of dark heavy-walled mycelium were found. There was a tendency for the hyphae to form in clumps (Fig. 16, *f*). Longitudinal sections showed that the tissues of the coleoptile adjacent to these clumps had been invaded to a considerable depth. The mycelium was seen to be massed between the coleoptile and the first leaf; infection of the latter had taken place (Fig. 15, *f*). Similarly, mycelium was seen in the axils of the coleoptile, first and second leaf, and growing point (Fig. 13B, *f*). This mycelium had invaded the tissues at these points and areas of collapsed tissue were evident. The fungus had been able to reach these points when the testa was broken by the expanding shoot (Fig. 13A, *t, sh*). This was the condition in the seedlings in which growth of the plumule stopped soon after it had started, but whose roots usually were fairly healthy. More extreme cases were found, in which shoot and roots were dead and filled with mycelium. Usually, the fungus was sporulating on the surface of the dead tissues. As has been stated, most of the seedlings emerged from the soil and continued to grow. Of these, a few produced one leaf and then died. An examination of these showed that the base of the coleoptile, and the leaves and growing point were severely lesioned, decayed, and full of mycelium. In almost all seedlings of this type, the root system was sound at first but soon died as the result of infection and starvation. The remainder of the seedlings developed more or less normally.



FIGS. 15 TO 18. Infection of barley by *H. sativum*. FIG. 15. Mycelium of *H. sativum* (f) massed between the coleoptile (col) and the first leaf (1l) and within the cells of the latter, in a seven day old seedling from naturally infected seed. $\times 350$. FIG. 16. Mycelium of *H. sativum* (f) massed on the surface and within the cells of the coleoptile (col) of a seven day old seedling of barley, from a naturally infected kernel. $\times 350$. FIGS. 17 AND 18. Cross sections showing lesions in the ventral and dorsal pericarp (pe), respectively, of spikelets inoculated with *H. sativum* seven days after flowering. The fungus (f) did not penetrate the chlorophyllous layer (c) of the pericarp or the inner integument (i). Al = aleurone; en = endosperm; ep.n = epidermis of nucellus; s.c(n) = sheaf cells (nucellus); tr = transchalazal



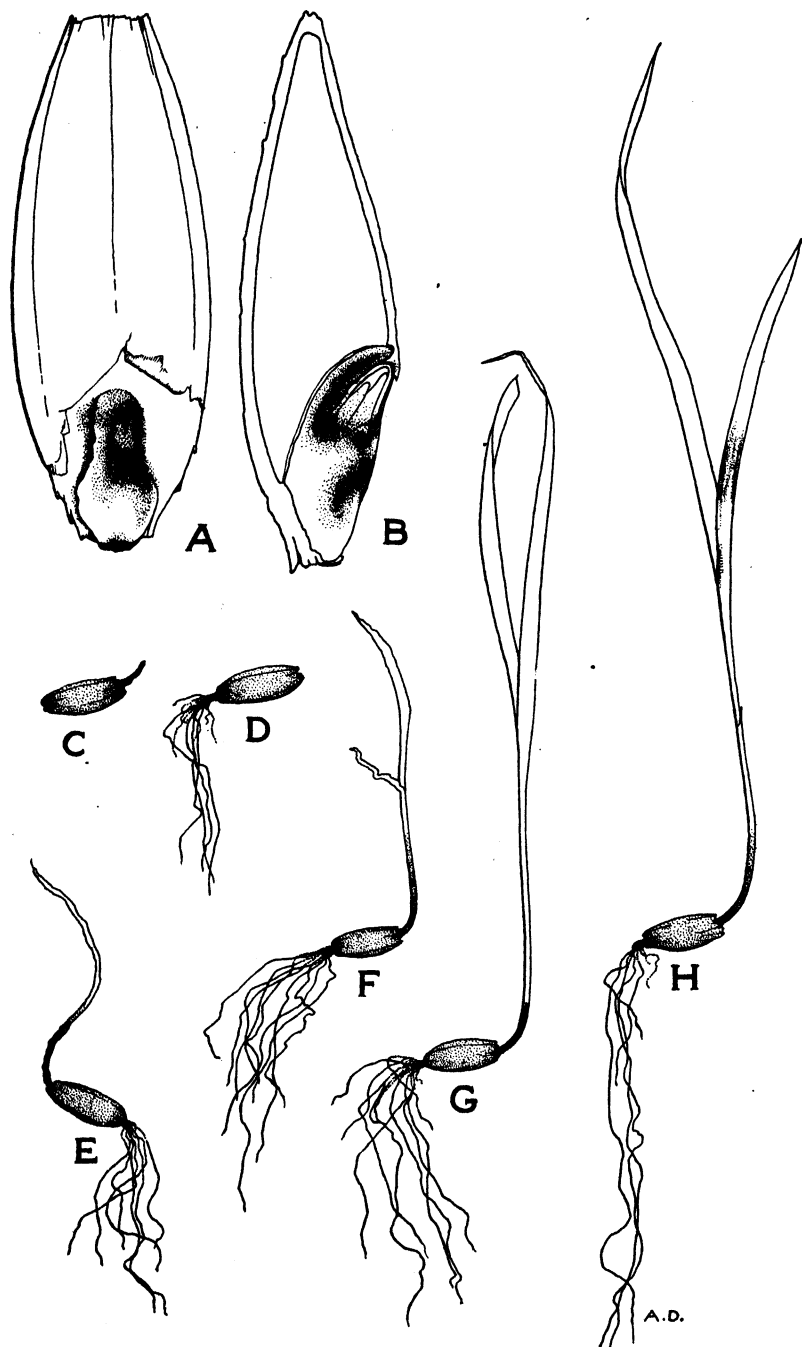


FIG. 19. Types of seedling injury by a seed-borne fungus, *H. sativum*. A, B, dorsal view and median section of embryo killed by the fungus, $\times 9$ approx.; C, D, E, pre-emergence blighting; F, postemergence blighting; G, severe basal lesioning and stunting; H, slight basal and foliar lesioning, $\times 1$ approx.

Their tissues were free of fungi entirely, or they were infected superficially and had surface lesions on the leaves, crown, subcrown internode¹, and roots.

The types of infected seedlings mentioned above are considered to represent five degrees of infection that may occur during germination when the seed is carrying *H. sativum*. These are as follows: (1) complete blighting of plumule and radicle at a very early stage (Fig. 19, A, B, C, D); (2) early blighting of the unemerged shoot with infection soon spreading to the roots (Fig. 19E); (3) postemergence blighting of shoot and roots (Fig. 19F); (4) stunting due to basal infection (Fig. 19G); (5) surface lesioning of shoot and roots with fairly strong growth (Fig. 19H).

Spread of the Fungus from the Seed into the Soil

When the diseased kernels were put in a moist chamber or on the surface of potato dextrose agar in a Petri dish at 24° C., hyphae of the fungus appeared within 24 hr., grew rapidly, and projected above the substratum into the moist atmosphere. It was impossible to demonstrate a similar growth in soil, except in an indirect manner. For this purpose, surface sterilized kernels of barley infected with *H. sativum*, and Ceresan treated² kernels to serve as healthy controls, were planted in moist sterile soil in Petri dishes. After four days the germinated kernels were uncovered and particles of the soil lining the pockets in which the kernels lay, were transferred aseptically to potato dextrose agar. At the same time particles of the same lot of sterilized soil were plated. The soil surrounding the diseased seeds produced colonies of *H. sativum*, whereas the check lots of soil particles did not do so. The inoculum that produced the colonies of *H. sativum* on agar could have been hyphae that penetrated the soil, or spores from the surface of the kernels. In order to demonstrate the presence of hyphae in the soil, surface sterilized diseased kernels were placed in contact with the ends of clean glass slides (4) in sterilized soil and the slides were examined four days later. Hyphae of a fungus had adhered to the glass and had grown for distances as great as 2.5 cm. from the end of the glass. These hyphae bore a few conidia of *H. sativum* on short branches.

The Effect of Seed Infection on Later Development of the Barley Plants

Experiment I

These studies were conducted in the greenhouse and in field plots. In the first experiment in the greenhouse, wooden forms with a removable wall were used, filled with a mixture of loam soil and sand. Untreated kernels of barley, infected with *H. sativum*, were planted 2 in. deep and the soil was kept moist. At intervals of one week, the side of the form was removed, and with a gentle stream of water, the soil was washed from the shoot and the upper half-inch of the roots, and the condition of these parts recorded. Then the side of the form was put in position and some of the same mixture of soil was placed around the plants and moistened with water. A summary of the data obtained is given in Table II.

¹ The internode above the point of attachment of the coleoptile.

² Ethyl mercury phosphate.

TABLE II
A COMPARISON OF THE DEVELOPMENT OF THREE GROUPS OF BARLEY SEEDLINGS FROM 38 SEEDS WHICH WERE INFECTED WITH *H. sativum*

Group	Age, wk.	Height, cm.	Serninal roots, No.	Crown roots, No.	Leaves, No.	Tillers, No.	Vigour	Lesioning		
								Coleoptile ¹ or subcrown internode ²	Roots	Leaves
A (25 plants)	1	14.5	5.7	0	2	0	All strong	23 sl.	4 ³	0
	2	20.7	6.0	0	3.7	1	24 strong, 1 weak	15 sl., 1 sv.,	9	8 sl.
	3	32.7	6.0	4	4	1.3	24 strong, 1 weak	14 sl., 2 m., 7 sv.	15	15 sl.
	4	43.0	6.0	8	7	2.1	24 strong, 1 weak	9 sl., 8 m., 7 sv.	18	First leaf dying on all plants
B (3 plants)	1	7.0	5.0	0	2	0	All stunted	1 sl., 2 sv.	1	2 sl.
	2	14.5	5.0	0	2	0	1 weak, 2 dying	1 m., 2 sv.	3	3 sv. on 1st leaf
	3	14.5	5.0	0	3	0	1 weak, 2 dead	3 sv.	3	1st and 2nd leaf dying
	4	14.5	5.0	0	3	0	3 dead	3 sv.	3	Dead
C (10 plants)	1	3.1	6.0	0	Non-emerged from coleoptile	0	All weak, distorted	2 sl., 8 sv.	1	Non-emerged from coleoptile
	2	3.1	6.0	0	0	0	All dead			

NOTE: sl. = slight; m. = moderate; sv. = severe.

¹ Coleoptile first week.

² Subcrown internode second, third, fourth weeks.

³ Number of plants with lesions on roots.

The results indicate that most of the plants (Group A) escaped severe primary infection and continued to grow strongly, although what infection there was spread gradually over the base of the plants and was becoming severe at the fourth week. This took the form of lesioning and rotting of the coleoptile, the cortex of the primary roots and subcrown internode, and blighting of the first leaf. Development of leaves and crown roots continued and the latter remained free of lesions. Generally, the growth of these plants appeared to be affected only very slightly by the fungous infection when the final data were taken. Group B included plants that were infected early but were able to emerge from the soil. Shoot and root development gradually stopped and the plants were dead by the fourth week. In Group C, 10 plants failed to emerge and died very quickly. It was observed, however, that the primary roots of these plants were quite healthy until the shoot died. Thus, under greenhouse conditions, 13 out of 38 plants from seed infected with *H. sativum* failed to survive beyond the fourth week, the majority of them having failed to emerge. The remainder, 25 plants, were growing strongly when the experiment was terminated, but many of them bore extensive basal lesions. The plants that died early showed signs of serious injury before the end of the first week whereas those that continued to grow were either free of infection or were infected lightly, at that time. It was observed, however,

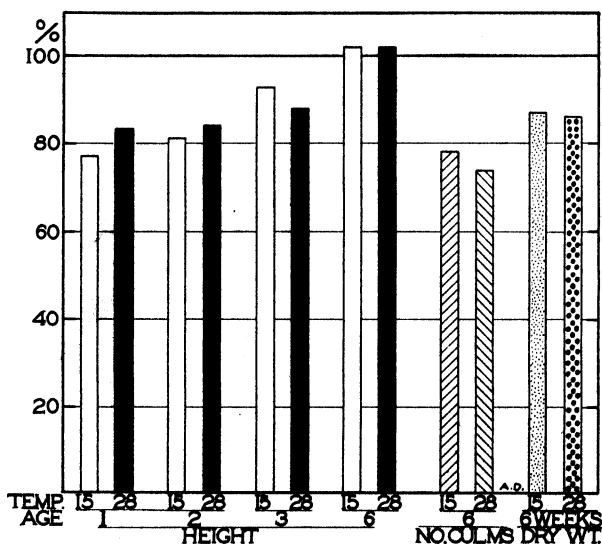


FIG. 20. Bar diagram comparing the development, under controlled conditions, of barley seedlings from healthy seed and from seed naturally infected with *H. sativum*. The data for the diseased seedlings are represented as percentages of equivalent data for the healthy seedlings which are shown by a horizontal straight line at 100%.

that in all cases where there were basal lesions on the coleoptile during the early stages of growth, the subcrown internode and roots became lesioned later. This infection spread rapidly on some plants. The further growth of the plants was related fairly closely to the extent of infection and the rapidity of its spread.

Experiment II

In another experiment conducted in the greenhouse, seed of the same diseased sample of barley was sown in soil in 1 gal. earthenware crocks that were suspended in water-baths. Untreated kernels were planted in one-half of the containers, and kernels treated with Ceresan were planted in the others. The soil moisture was maintained at 60% of its moisture holding capacity by regular weighings, and temperatures of 15 and 28° C. were maintained in water-baths. Ten kernels were sown 1½ in. deep in each container. The height of the plants was measured at one, two, and three weeks after sowing, and again at the sixth week, when the number of culms and dry weight also were recorded. The data for the plants from untreated seed are represented graphically in Fig. 20, as percentages of the corresponding data for plants from treated seed, which are rated at 100%.

Under controlled greenhouse conditions where the plants from untreated seed did not have to compete with plants from treated seed, there was a gradual recovery in height by the diseased plants, following an initial set-back. However, the plants from untreated seed had fewer culms and were lighter in weight than those from treated seed. Sallans (19) found that under favourable conditions wheat seedlings showed definite signs of recovery from an initial severe set-back by *H. sativum*.

Experiment III

An experiment was conducted in the field with seed from the same sample sown in rows six inches apart. A plot consisted of one row of untreated seed, and one row of treated seed, and it was separated from the next plot by a row of treated seed. There were 16 plots arranged in four by four Latin square. This provided sufficient material for four dates of harvesting, each having four replicates. A count of the emerged seedlings was made 14 days after seeding. The mean emergence in the 16 untreated rows was 39% and that in the 16 treated rows 94%. At this time all of the plants in four replicates were pulled to be used for measurement of height and dry weight, and for making counts of culms and observing disease symptoms.

The plants in the treated rows of the remaining plots were thinned so as to leave the same number as had emerged in the untreated rows. The rows separating the plots were not thinned and their heavy growth provided strong competition for the thinned rows. Quadruplicate plots were harvested at

28, 42, and 80 days. The data from the untreated rows are represented graphically in Fig. 21, as percentages of the corresponding data from the treated rows, which are rated at 100%.

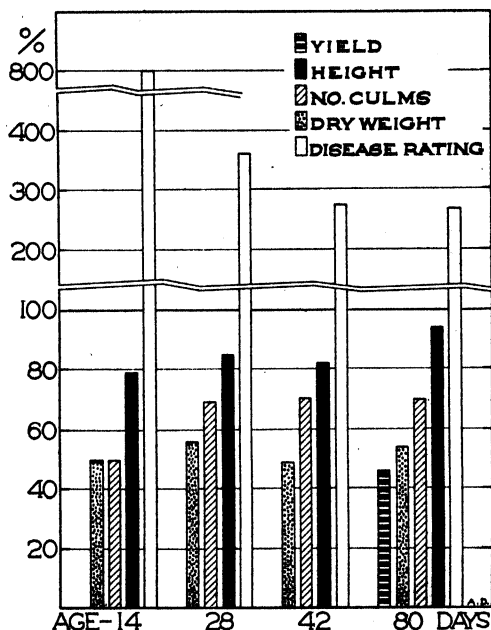


FIG. 21. Bar diagram comparing the development, under field conditions, of healthy barley seedlings and seedlings from seed infected with *H. sativum*. The data for the diseased seedlings are represented as percentages of equivalent data for the healthy seedlings, which are shown by a horizontal straight line at 100%.

Under field conditions, where there was strong competition for moisture with neighbouring unthinned rows of healthy plants, the plants from untreated seed were weaker throughout the season than those from treated seed. The 14-day old diseased plants had a high disease rating¹ and were stunted and light in weight. As time passed they compared more favourably with the healthy plants. The latter became lesioned as the season progressed, but maintained their superiority in height, number of culms, and weight. Their yield was more than twice as great as the yield from the plants in the untreated rows. These results indicate that under field conditions where there was strong competition for moisture, the effects of an early severe attack by *H. sativum* on barley seedlings persisted, and were only partially overcome by the plants.

¹ Rated as follows: blighted, 10; severely lesioned, 4; moderately lesioned, 2; slightly lesioned, 1.

$$\text{Disease rating} = \frac{\text{sum of ratings}}{10} \times \frac{100}{\text{number of plants}}$$

The results given above show that the fungus, *H. sativum*, when borne on the seeds, was capable of causing severe injury to the barley seedlings under a fairly wide range of temperature conditions, in the presence of sufficient moisture to give good growth. The most obvious and serious injury was seedling blight. However, light infection also occurred and spread over the base of the plant, involving the roots, subcrown internode, and leaves. The reaction of the individual seedlings to infection varied, but in general, a severe early infection caused stunting and early death. In most cases there was considerable recovery from the effects of infection. This occurred where competition for moisture was not severe. Even when the extent of seed infection was known it was impossible to predict the amount of injury which would be caused by the fungus. This seemed to be a complex result of various factors affecting host and parasite.

Discussion

The relationship between the barley kernel and a fungus parasite borne in and upon the seed coverings is much more intimate than the relation between the kernel and the same parasite in soil. Proximity in itself should make a difference, but there are physiological, nutritional, and probably antibiotic factors also which are quite different. A fungus within the seed coat has certain food substances immediately available on germination of the seed, which are not available to soil fungi. In its relation to other microorganisms, the seed-borne fungus of barley is protected to a greater extent than the soil-borne fungus. Competition for food is not so acute, and antibiosis probably is less effective because of the hard seed coverings.

Barley kernels may become infected with *H. sativum* during maturation. The commonest forms of inoculum are air-borne spores and fragments of mycelium. These may come from neighbouring crops, grasses, or crop refuse. The time of inoculation is important, because the survival and further development of the kernels depends upon it. It has been shown that spikelets inoculated at flowering time usually are killed outright, while later inoculation may cause only shrivelling and discoloration. The opportunities for infection by air-borne conidia become limited by the changes in the tissues during development. The floral glumes become hard in texture and after the third week following flowering adhere to the pericarp. This is an important barrier to the entry of inoculum. In addition to this, the caryopsis steadily develops a resistant membrane, the testa, which becomes more resistant as time goes on. The spread of the fungus is likewise limited by the natural collapse of the tissues, with a consequent draining of organic nutrient materials and moisture which might be used by the fungus.

The fungus does not live in complete compatibility with the tissues of the barley kernel. Some host cells attempt to prevent penetration by formation of callosities. Those that become colonized eventually die and collapse. The mycelium in the dead cells remains viable and germinates to produce hyphae that infect other cells, or forms conidiophores and conidia. The cells

that are killed, singly or in groups, become discoloured and constitute lesions. Strands of the fungus may, however, remain on the surface of cells or in intercellular spaces without any apparent harm to the cells. The surface mycelium usually is thick-walled and its protoplasm may become vacuolated.

The distribution of the fungus in the tissues bears a direct relation to infection of the seedling. The plumule and radicle of the embryo rupture the testa and pericarp of the kernel and remain in contact with diseased tissue. This includes pericarp, lodicules, and glumes. Rupture of the testa permits spread of the fungus from the pericarp to the base of the plumule and radicle. This may be followed by rapid invasion of the growing point, lateral bud, and primary roots. Usually, however, the fungus requires considerable time to penetrate the coleoptile and primary leaves in order to reach the growing point. Similarly, the coleorhiza blocks the progress of the fungus towards the roots for a short time. Thus, the speed of penetration of the outer sheaths of the embryo determines to some extent the fate of the shoot and roots. These may be killed or may continue to grow and bear lesions in their cortical tissue. These lesions provide inoculum for secondary infection.

A variable amount of stunting of infected seedlings was evident from the beginning of their development. This was caused by destruction of certain tissues and probably by toxic action of the fungus and competition for food. Recovery took place gradually if nutrient materials and moisture were available but many of the infected plants suffered permanent injury in the form of reduced root growth and tillering. The results of a field experiment under dry conditions were indicative of the importance of competition for moisture. In that experiment, unthinned rows of healthy barley flanked the thinned rows of diseased and healthy barley. The result was that the diseased plants were much inferior to corresponding healthy plants throughout the season.

Acknowledgments

The author is indebted to Dr. P. M. Simmonds, Dr. D. L. Bailey, and Dr. H. B. Sifton for guidance during the course of these investigations. The assistance of Mr. A. W. Davey in preparation of some of the figures is gratefully acknowledged.

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ENVIRONMENTAL RELATIONSHIPS IN A SEED-BORNE DISEASE OF BARLEY CAUSED BY *HELMINTHOSPORIUM SATIVUM* PAMMEL, KING, AND BAKKE¹

BY H. W. MEAD²

Abstract

The experiments described in this article show that the greatest seedling injury occurs under conditions that are unfavourable to the host, i.e., high temperature and excessive moisture or low temperature and scant moisture; that the greatest recovery takes place at 15 to 18° C. in moist soil. They further show that packing and fertilization of the soil increase the amount of infection, though fertilization may increase the dry weight of the seedlings; that the microflora of the soil has little influence upon the seed-borne parasite; that reduction of the oxygen content of a nutrient solution and of soil from 21% to 10% depresses the growth of the seedlings and the amount of infection; that the addition of 1% of carbon dioxide to the atmosphere of soil increases the amount of seedling infection. From these results the conclusion is drawn that barley that is infected with *H. sativum* should be sown in cool, moist, well aerated soil.

Introduction

In a previous article (13) it was shown that *Helminthosporium sativum* Pammel, King, and Bakke may parasitize barley spikelets. When infected mature kernels were sown, the resulting seedlings showed evidence of blighting, malformation, stunting, and lesioning, according to the conditions of growth. This article deals with experimental work conducted in the greenhouse and in field plots for the purpose of studying the effects of certain environmental factors on the disease, as found in two samples of barley, one from Manitoba, the other from New Brunswick.

Environmental Factors

A. Soil Microflora

There is little information on the effect of the microflora of the soil on the development of seedling blight or root rot arising from naturally infected seed. If the soil microflora prevents or appreciably inhibits seedling injury which usually arises from infected seed, it would be of considerable economic importance. Christensen (2) found that there were no differences in germination or amount of seedling injury when barley seed naturally infected with

¹ Manuscript received in original form January 26, 1942, and as revised, July 10, 1942.

Contribution No. 713 from Division of Botany and Plant Pathology, Science Service, Dominion Department of Agriculture, Ottawa, Canada. Based on part of a thesis submitted to the University of Toronto in 1942 in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Helminthosporium or *Fusarium* was planted in sterilized and non-sterilized soil. Similarly, the addition of *Trichoderma lignorum* (Tode) Hanz and several other fungi and bacteria to naturally infected barley seed did not inhibit or delay the parasitic action of the seed-borne parasites. Hynes (10) showed that under some conditions biological antagonism was not of importance in limiting injury from *H. sativum*. Henry and Campbell (9) studied the effect of sowing flax seed that was naturally infected with *Polyspora Lini* Laff. in sterilized and natural soil. A consistent and marked reduction in disease was evident in the natural soil as compared with the sterilized soil. Indeed, the severity of infection was reduced more by antibiotic action than by seed treatment. However, in preliminary tests with wheat naturally infected with bunt fungi, there was no reduction in infection in natural soil as compared with sterilized soil.

Experiment I

Greenhouse tests were conducted with the New Brunswick sample of barley. It was grown in sterilized and natural soil to determine any differences in infection due to soil microflora. The seed was sown in loam soil in six-inch pots. One-half of the pots contained sterilized soil, the other half natural soil. Similarly, untreated seed and Ceresan-treated seed were used. The pots were arranged in a four by four Latin square on the greenhouse bench and were kept moist by surface watering. The results of two tests are summarized in Table I.

TABLE I

EFFECT OF SOIL STERILIZATION ON EMERGENCE AND INCIDENCE OF DISEASE IN BARLEY FROM SEED INFECTED WITH *H. sativum*

Seed	Soil	Emergence, %	Disease rating, %
Healthy	Natural	98.5 ± 1.6	2.2 ± 1.7
Healthy	Sterilized	98.0 ± 1.4	2.9 ± 1.4
Diseased	Natural	67.2 ± 9.9	46.1 ± 12.7
Diseased	Sterilized	71.5 ± 14.4	42.9 ± 10.6

The data were examined statistically by Fisher's analysis of variance method as outlined by Goulden (6). The differences in the amount of disease in sterile and natural soil were not significant.

B. Temperature and Moisture

Dosdall (3) studied the temperature relations of *Helminthosporium sativum*. She found that mycelial development occurred over a wide range, 1° to 37° C., with the best growth near 28° C. Germ tubes penetrated coleoptile and leaf tissue from 12° to 34° C. but severe infection and rapid development occurred only between 22° and 30° C. Above 30° C. the development of lesions seemed to be checked. Jones, Johnson, and Dickson (11) found that the cereals, wheat and barley, grew best at relatively low temperatures, 12° to 16° C.

Dosdall (3) found that the percentage and severity of infection in barley seedlings, as determined by plant growth, increased as the amount of soil moisture was increased. Her results also indicate that seedling barley plants suffer most from root infection in soil containing maximum or minimum extremes of moisture. McKinney (12), working with wheat, found that high soil temperatures (28° to 32° C.) and high soil moisture content (55 to 65% moisture holding capacity) favoured infection. When the soil temperature was high, high soil moisture content was most favourable for infection, while at low temperatures, parasitic attack was greater at lower soil moistures. Extremely low soil moistures were not favourable to infection at any temperature, and at extremely high and low temperatures the moisture curves for infection were irregular.

Experiment II

Six combined soil temperature and moisture tests were conducted in soil in the greenhouse. In Tests 1 and 2 the soil was sterilized; in Tests 3, 4, 5, and 6 it was not sterilized. Two moisture levels were used, these being 30 to 35% (low) and 50 to 55% (high) and they were maintained by regular weighings of the containers and surface watering. In Tests 1 and 2, the sample grown in Manitoba was sown and the New Brunswick sample was used in Tests 3, 4, 5, and 6. In Tests 3 and 4 the air and soil temperatures were controlled alike; in the other tests the air temperature was that of the greenhouse. The results of the tests are given in Table II.

The results obtained from the two samples of infected barley were quite different. This might be expected, because different varieties, possibly infected with different strains of the fungus were grown in sterilized and natural soil respectively. In Tests 1 and 2, where the Manitoba sample was used, there was little infection at 12° C., in either the high or low moisture series. The amount of infection increased as the temperature was raised to 21° C., but it was affected very little by increase in moisture. The strain of *H. sativum* present on this sample of seed was not particularly virulent. It caused very little seedling blight and only moderate lesioning. The strain of *H. sativum* on the New Brunswick sample was very virulent. It caused considerable seedling blight and from moderate to severe lesioning at 12°, 18°, and 25° C. whether the moisture content of the soil was low or high. Moisture was a limiting factor, however, at 32° C.; the greatest amount of blighting and lesioning occurred in the high moisture series. Because the soil in Tests 3, 4, 5, and 6 was not sterilized, control tests using disease-free susceptible barley were conducted, and only a trace of lesioning occurred on a few plants. It is evident then that the injury done to the seedlings was caused by the seed-borne fungus.

Experiment III

Field tests were conducted in 1941 with the New Brunswick sample of barley for the purpose of studying the effect of early, medium, and late sowing on emergence. The seed was sown in rows six inches apart in four blocks with

TABLE II
SUMMARY OF EMERGENCE AND LESIONING IN BARLEY FROM SEED INFECTED WITH *H. sativum* GROWN UNDER CONTROLLED CONDITIONS

Test	Soil moisture	12° C.		16° C.		18° C.		21° C.		25° C.		32° C.	
		Emerged, %	Lesioned, %	Emerged, %	Lesioned, %	Emerged, %	Lesioned, %	Emerged, %	Lesioned, %	Emerged, %	Lesioned, %	Emerged, %	Lesioned, %
1	Low	98	6	94	26			98	48				
	High	100	2	92	30			96	34				
2	Low	94	8	100	26			94	40				
	High	100	6	98	36			90	38				
3	Low	90	50							100	80	100	20
	High	90	50							80	80	90	80
4	Low	80	20							90	90	100	20
	High	100	30							90	90	100	30
5	Low	86	60			82	62			84	60	52 ¹	12
	High	86	68			80	70			90	78	92	40
6	Low	86	76			80	66			84	56	94	20
	High	86	72			80	78			92	88	84	76

¹ Reduction in emergence caused by uneven moisture distribution.

four replicates in each block. Rows containing some of the same seed treated with Ceresan were included in the tests. The difference in emergence between these rows and those containing untreated seed was ascribed to blighting by the seed-borne fungus, *H. sativum*. The results of the field tests are given in Table III.

TABLE III

THE EFFECT OF DATE OF SEEDING UPON THE EMERGENCE OF BARLEY FROM SEED INFECTED WITH *H. sativum*

Date of seeding	Emergence		Necessary difference*
	Untreated, %	Treated, %	
April 26	56.7	88.9	14.9
May 30	70.0	93.3	11.8
June 30	43.6	85.6	24.3

* Necessary difference, 5% level of significance, between untreated and treated series.

In the field the most severe blighting occurred during the week following the third date of seeding when the mean daily soil temperature was 20.1° C. This temperature is higher than the optimum for barley seedlings but lower than the optimum for the fungus. There was more blighting following the first date of seeding than following the second. It appears probable that the soil conditions for host development were most favourable during the period following the second date of seeding and that blighting was less severe because of that condition.

In general, the greenhouse and field experiments indicated that when a virulent strain of *H. sativum* was present on barley seed, it infected the seedlings throughout a wide range of temperature and moisture. Where there was just sufficient moisture for strong germination, the fungus was active at low and moderate temperatures, but was inhibited at very high temperature (above 30° C.). An increase in soil moisture made little difference at low and moderate temperatures but increased damage from infection at high temperature. The results suggest that a crop of barley would suffer least damage from *H. sativum*, when borne on the seed, if the seed were sown when soil conditions, moisture and temperature, favoured the barley plants.

C. Firmness of Seed Bed

Experiment IV

Observations made while removing infected barley seedlings from soil indicated that the shoots of certain plants were weak, and being unable to push through the soil, died in a short time. Other severely infected plants were sufficiently strong to emerge, produce one or two leaves, and survive for two or three weeks. A greenhouse test was planned to study the effect of packing the soil on emergence, stunting, and blighting in the case of barley

seed infected with a virulent strain of *H. sativum*. Untreated and treated seed was sown two inches deep in moist loam soil in six-inch pots. The soil in one-half of the pots was pressed firmly after the seed was sown. There were four replicates of each treatment. The pots were set in a four by four Latin square arrangement on the greenhouse bench, and were kept moist by surface watering. The greenhouse temperature range was from 16° to 26° C., with a mean of about 22° C. The data are summarized in Table IV and an analysis of the emergence is given in Table V.

TABLE IV

EFFECT OF PACKING SOIL ON EMERGENCE AND DISEASE IN BARLEY FROM HEALTHY SEED, AND SEED INFECTED WITH *H. sativum*

Seed	Soil	Test	Emergence, %	Stunted, %	Blighted, %
Healthy	Unpacked	1	98	1	2
		2	98	1	2
		3	96	2	4
Healthy	Packed	1	99	0	1
		2	97	0	3
		3	100	2	0
Diseased	Unpacked	1	80	21	20
		2	84	10	16
		3	75	10	25
Diseased	Packed	1	65	27	35
		2	73	13	27
		3	70	5	30

TABLE V

ANALYSIS OF VARIANCE OF EMERGENCE DATA GIVEN IN TABLE IV

Source of variance	D.f.	Mean square
Replicates	3	5.33*
Disease	1	420.00**
Packing	1	16.00**
Disease × packing	1	25.00**
Error	41	1.98

Significance, * = 5% point; ** = 1% point.

The analysis indicates that packing of the soil caused a significant reduction in emergence of diseased seed. When the soil was packed, the amount of seedling blight and stunting increased. Hynes (10) found that the *H. sativum* and *F. culmorum* type of root rot of wheat in a district of New South Wales in 1932 was extensive on fallow that had a limited cultivation previous to

sowing, while in those cases where the fallow had been cultivated four to six times during the eight or nine months prior to seeding, the disease was slight. He attributed the partial control in the latter case to a combination of biological control and increased crop vigour. Hynes (10) and Christensen (2) considered that antibiosis plays a small role in limiting the amount of injury from *H. sativum*, especially where the fungus is seed-borne. The results of Experiment IV support this view. It seems, therefore, that the increase in injury by *H. sativum* when the soil is packed cannot be ascribed entirely to suppression of biological activity. The writer considers that packing exerted an adverse mechanical effect upon weak seedlings, the influence of which was greater than its physiological effect upon the fungus or factors that affect the fungus. A characteristic feature of seedling injury by this fungus is weakening and distortion which limits the ability of the seedlings to emerge. Any factor that increases the resistance of the soil tends to reduce the emergence. As shown in Table IV, packing of the soil under greenhouse conditions caused reduction in emergence when diseased seed was sown.

D. Fertilization of the Soil

Dosdall (3) found from field experiments, designed to show the effect of fertilizers on infection in wheat and barley by *H. sativum* that there was no correlation between the amount of disease and any particular fertilizer. Russell and Sallans (18) in studying the incidence of common root rot on wheat fertilized with phosphate fertilizers, found that fertilized wheat usually exhibited a somewhat higher disease rate than unfertilized wheat. There was a tendency for this to occur only where there was an increase in yield also. Greaney (7) concluded that deficiencies of phosphorus failed to influence the severity of infection of wheat seedlings by *Fusarium culmorum* (W. G. Smith) Sacc., while an excess of phosphorus appeared to accentuate it. Broadfoot and Tyner (1) concluded that extremely small concentrations of phosphorus had no effect on root rot of wheat caused by *H. sativum*, and excess phosphorus produced no significant reduction in the amount of disease. Vanterpool (22, pp. 234-241) found that addition of phosphate fertilizers to "browning" soil increased the dry weight of wheat plants and the number and length of crown roots. The ratio of healthy to diseased roots was increased also.

Experiment V

The effect of adding ammoniated superphosphate (2-19-0) fertilizer to the soil when barley infected with *H. sativum* was sown, was studied in the greenhouse. A portion of the seed was treated with Ceresan to represent healthy seed. The seed was sown in loam soil in six-inch pots and 0.5 gm. of the fertilizer was mixed with the soil surrounding the seeds. The pots were set up in a Latin square formation including four replicates and four treatments. Data on emergence, disease rating, and dry weight are presented in Table VI and the analyses of variance are given in Table VII.

TABLE VI

EFFECT OF FERTILIZATION OF SOIL ON EMERGENCE, DISEASE, AND DRY WEIGHT OF BARLEY SEEDLINGS FROM HEALTHY SEED AND SEED INFECTED WITH *H. sativum*

Seed	Soil	Test	Emergence, %	Disease rating, %	Dry weight per plant, mg.
Healthy	Unfertilized	1	99	0	20.0
		2	98	0	20.4
Healthy	Fertilized	1	98	0	23.1
		2	97	0	21.0
Diseased	Unfertilized	1	80	12.3	15.8
		2	77	11.2	15.1
Diseased	Fertilized	1	73	16.0	16.5
		2	70	12.0	18.4

TABLE VII

ANALYSES OF VARIANCE OF DATA SUMMARIZED IN TABLE VI

Source of variance	D.f.	Mean squares	
		Emergence	Dry weight
Replicates	3	6.25	98.54
Disease	1	264.50*	12720.13*
Fertilization	1	8.00	1682.00**
Disease × fertilization	1	4.50	544.50
Error	25	2.79	302.84

Significance: * = 5% point; ** = 1% point.

Emergence of diseased seed in the fertilized pots was lower than in the unfertilized pots but the difference was not significant. It was caused by increased pre-emergence blighting. The increase in dry weight of the seedlings by fertilization was significant. The results show that infection of barley seedlings by *H. sativum* may be more severe in the presence of a phosphate fertilizer than without it, but that the surviving seedlings grow more vigorously than unfertilized seedlings.

E. Soil Atmosphere

Russell and Appleyard (17) found that the soil air commonly contains less oxygen and more carbon dioxide, usually also more nitrogen than atmospheric air but the differences are often small. However, the soil air is not constant in composition but changes somewhat from day to day and to a greater extent from season to season; the latter may be so great as to mask altogether the local fluctuations. Whatever the history of the soil, its atmosphere in spring, and to a less extent in autumn was characterized by high

amounts of carbon dioxide while in summer and winter the amounts were much lower. The temperature and moistness of the soil affect the composition of the soil atmosphere as do manuring and cropping. The results of analysis of four arable Rothamstead soils gave the following percentages by volume in the soil atmosphere: carbon dioxide, 0.25%; oxygen, 20.6%; nitrogen, 79.12%. Atmospheric air was found to contain 0.03% carbon dioxide, 20.9% oxygen, and 79% nitrogen. Thus the chief difference was the amount of carbon dioxide in air and in soil. It reached 2% in manured soils during the biologically active season. Carbon dioxide content of garden soils may sometimes be so high as to be detrimental to the root development of some common species (15). Few plants can survive long in soil or water low in oxygen. An important source of oxygen is rain water which is highly charged with the gas (23). Hall, Brenchley, and Underwood (8) found that the rate of root growth and top growth in both soil and water culture could be increased by pulling a stream of air through the medium. In an experiment, they found that the average dry weight of barley plants in non-aerated water cultures was 1.31 gm. and in aerated cultures, 2.12 gm. According to Miller (14) depletion of the oxygen supply of the soil interferes with the process of respiration in the roots, through injury to the protoplasm, causing it to fail as an absorbing organ. Stiles and Jörgensen (20) found that aeration of a nutrient solution increased the rate of growth of barley. Garrett (5) found that by aerating acid soils, they could be made as favourable as alkaline soils for the growth of *Ophiobolus graminis* Sacc. along the roots of wheat seedlings. This finding was in agreement with his theory that the growth of the fungus in acid soils is retarded by the accumulation of respiratory carbon dioxide. Fellows (4) studied the effect of a scarcity of oxygen and an excess of carbon dioxide in the atmosphere surrounding pure cultures of *O. graminis*. In a liquid medium, growth diminished gradually as the oxygen concentration decreased; on a solid medium marked diminution did not occur until oxygen was below 6%. A very small percentage of oxygen greatly reduced growth. The fungus grew well on both media when the carbon dioxide content was varied, although at the highest carbon dioxide concentration (18.02%), some diminution in growth occurred. Fellows believed that the variations in carbon dioxide and oxygen as found in arable soils are not great enough to affect materially the growth of *O. graminis*. Owen, Small, and Williams (16) found that an increase of the carbon dioxide concentration of the atmosphere in the greenhouse did not affect germination of tomatoes, but did increase the yield. The plants showed an increased susceptibility to *Colletotrichum atramentarium* (B. and Br.) Taub., in an atmosphere rich in carbon dioxide.

Some experiments were conducted in the laboratory to test the effect of lowering the supply of oxygen and of increasing the supply of carbon dioxide in soil or nutrient solution on the growth of barley seedlings and on the severity of infection of these seedlings by the seed-borne fungus, *H. sativum*. In addition, the effect of an atmosphere, low in oxygen, on the growth of *H. sativum* in culture was studied.

Experiment VI

In this experiment the barley was grown on perforated cork disks which floated on a nutrient solution (19) which was renewed constantly. One set of containers was aerated with a stream of air, the other set with a mixture of nitrogen and oxygen in the proportion 9:1 by volume. In each case the solution was stirred constantly. The containers were set in a water-bath which was held at constant temperature.

TABLE VIII

THE EFFECT OF REDUCING THE OXYGEN SUPPLY IN A NUTRIENT SOLUTION, UPON GROWTH AND DISEASE INCIDENCE IN BARLEY FROM HEALTHY SEED AND SEED INFECTED WITH *H. sativum*

	Seed	Aerated with:		Necessary difference*
		Air	N ₂ + O ₂ (9 : 1)	
Germination (9 seeds)	Healthy Diseased	9 7	8 7	
Height (cm.)	Healthy Diseased	9.3 8.5	6.5 2.0	3.6 5.1
Total root length per plant (cm.)	Healthy Diseased	20.0 19.5	17.6 7.2	17.8 11.3
No. seedlings infected	Healthy Diseased	0 7	0 0	

* Necessary difference, 5% level of significance between treatments.

In this experiment, general growth of seedlings from both healthy and diseased seed was depressed by a scarcity of oxygen, but the effect was greatest in the case of diseased seed. Similarly, infection of the seedlings by the seed-borne fungus (*H. sativum*) was suppressed. This was determined by direct examination of coleoptile tissue for evidence of penetration and for lesions.

Experiment VII

In this experiment natural soil was used instead of a nutrient solution. Diseased kernels of barley were sown in moist, sifted, unpacked soil in glass tubes 12 in. long and 1 in. in diameter. These tubes were connected in series and one set was aerated with air, the other with a 9:1 mixture by volume, of nitrogen and oxygen. The air and nitrogen-oxygen mixture were first passed through Chamberland filters, then through sterile water. The tubes containing the soil and seeds were immersed in a water-bath, the temperature of which was 21° C. Data on germination, growth, and disease symptoms are summarized in Table IX.

These results are similar to those given in Table VIII. They show that a scarcity of oxygen depressed the general growth of the barley plants and decreased the amount of infection by the seed-borne fungus.

TABLE IX

THE EFFECT OF REDUCING THE OXYGEN SUPPLY IN SOIL, UPON GROWTH AND DISEASE INCIDENCE IN BARLEY FROM SEED INFECTED WITH *H. sativum*

	Aerated with:		Necessary difference*
	Air	N ₂ + O ₂ (9 : 1)	
Germination (15 seeds)	15	15	
Mean height (cm.)	9.2	3.3	3.7
Total root length per plant (cm.)	26.1	18.6	20.5
No. seedlings infected:			
Shoot	11	6	
Root	13	1	

* Necessary difference, 5% level of significance.

Experiment VIII

The same apparatus, with tubes of natural soil, was used to test the effect of increasing the amount of carbon dioxide in the soil atmosphere on seedling growth and seed-borne infection. One lot of tubes was aerated with air, the other with a mixture of 99% air and 1% carbon dioxide, by volume. Healthy and diseased lots of seed were used, and the temperature was 21° C. The results are given in Table X.

TABLE X

THE EFFECT OF INCREASING THE CARBON DIOXIDE CONTENT OF SOIL, UPON GROWTH AND DISEASE INCIDENCE IN BARLEY FROM HEALTHY SEED AND SEED INFECTED WITH *H. sativum*

	Seed	Aeration with:		Necessary difference*
		Air	Air + 1% CO ₂	
Germination	Healthy (10 seeds)	10	10	
	Diseased (30 seeds)	28	29	
Height (cm.)	Healthy	11.5	11.6	7.6
	Diseased	11.5	9.7	10.8
Total root length per plant	Healthy	49.3	48.6	11.3
	Diseased	37.4	30.2	27.8
No. seedlings infected	Healthy	0	0	
	Diseased	13	19	

* Necessary difference, 5% level of significance between treatments.

The amount of carbon dioxide in the soil atmosphere in the air plus carbon dioxide series was considerably greater than that which is present in air or in ordinary soil atmosphere. The results show a slight reduction in height and in root development and a moderate increase in the number of plants infected.

Experiment IX

This experiment was conducted in the laboratory. In it, *H. sativum*, originating from disks of agar bearing the mycelium, and from barley kernels inoculated with the fungus during maturation was exposed to two kinds of atmosphere, at constant temperature. Two sets of agar cultures (2% potato dextrose agar) were placed in sealed containers through which air and a 9:1 mixture of nitrogen and oxygen, respectively, were passed. Both gases were filtered through cotton and a Chamberland filter and humidified by passage through sterile water. The covers of the Petri dishes were removed before they were placed in the chambers, in order to expose the fungus directly to the gas. The chambers were set in a water-bath, the temperature of which was maintained at 17° C. On the fifth day, the diameter of the colonies was measured and general observations on the thickness of the aerial mycelium and the progress of sporulation were made. These data are given in Table XI.

TABLE XI

THE EFFECT OF REDUCING THE OXYGEN CONTENT OF THE ATMOSPHERE UPON THE GROWTH OF *H. sativum* ON POTATO DEXTROSE AGAR

Inoculum	Diameter of colonies, cm.		Necessary difference*
	In air	In N ₂ + O ₂ (9 : 1)	
Mycelium	3.12	3.00	0.62
Diseased seeds	2.11	1.95	0.14
Sporulation	Well advanced	Trace	

* Necessary difference, 5% level of significance between treatments.

There was little difference in the radial growth under the two sets of conditions, but the mycelial mat was noticeably thicker in air than in the nitrogen-oxygen mixture, and it was sporulating more freely.

The results of the experiments that deal with the effect of changes in atmosphere, because of their limited scope, are considered to be indicative rather than conclusive and to agree in some respects with those of other investigators. They indicate that changes in the amount of oxygen and carbon dioxide in the atmosphere in which barley kernels are germinating may affect the growth of the seedlings themselves and the development and parasitism of a seed-borne fungus. Generally reduction in oxygen caused stunting of the seedlings and reduced the activity of the fungus and this is interpreted to be the result of slower metabolism of both organisms. There was some reduction in seedling growth and a slight increase in the activity of the fungus when the amount of carbon dioxide in the atmosphere was increased to 1.25%. This appears to be similar to a condition in soil that is only moderately well aerated. The top and root growth of plants from diseased kernels was reduced more by lack of oxygen than that of the plants

from healthy kernels. The interpretation placed on this is that the kernels were weaker to begin with, because their embryos competed with a fungus for food supplies during maturation. In addition, there is the possibility that this fungus excreted an injurious toxin which might pass the semi-permeable layer of the kernel. Vanterpool (21) found that *H. sativum* on certain media, produces a substance toxic to wheat.

Discussion

When this disease was studied under controlled conditions, it was found that certain environmental factors affected its intensity and that these factors affected both host and parasite. Temperature and moisture were closely associated. For instance, high temperature (32° C.) in itself was not limiting, but when associated with a lack of moisture it reduced the amount of infection. Seedling growth was poor at that temperature also. The fungus caused severe blighting at temperatures from 10° to 30° C. as long as there was sufficient moisture for good seedling growth. The strongest plants and best stand were obtained from diseased seed when the temperature was between 15° and 18° C. This temperature is closer to the optimum for barley than it is to the optimum for *H. sativum*. It has been concluded that seeding of diseased barley might better be done when conditions favoured the plant, i.e., when temperature was about optimum and moisture conditions good.

Well tilled and well aerated soil were more favourable to the diseased barley than packed or water-logged soil, or soil poor in oxygen or containing an excess of carbon dioxide. This was interpreted to mean that the favourable growing conditions were primarily beneficial to the barley seedlings rather than exerting an antibiotic effect on the fungus through intense biological activity. Enrichment of the soil with a phosphate fertilizer increased the disease but increased the vigour of the seedlings also, the net result being a slight reduction in stand and a slight increase in dry weight.

Acknowledgments

The author is indebted to Dr. P. M. Simmonds, Dr. D. L. Bailey, and Dr. H. B. Sifton for guidance during the course of these investigations. The assistance of Dr. B. J. Sallans in analysing the results is gratefully acknowledged.

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NON-STERILE SOIL AS A MEDIUM FOR TESTS OF SEED GERMINATION AND SEED-BORNE DISEASE IN CEREALS¹

BY J. E. MACHACEK² AND H. A. H. WALLACE³

Abstract

Experiments with non-sterile soil in large seed beds showed that such soil, if maintained in proper condition, could be used with satisfactory results for tests of seed germinability and of certain kinds of seed-borne diseases in cereals.

Non-sterile soil was more easily handled than autoclaved soil and it did not require fresh preparation for each planting.

The amount of soil-borne infection in the seedlings was negligible when friable, non-sterile soil was kept moist and at 20° C.

A comparison of the results from 120 different lots of seed showed that a test of seed in non-sterile soil was equal in some respects, and superior in others, to a test on moist paper or to a test on nutrient agar in Petri dishes as a means of measuring seed germinability, seed-borne disease, and physical injury to the seed. With barley seed, a plating test, in addition to the soil test, was found necessary to indicate the amount of seed-borne infection due to *Helminthosporium teres*.

A table of tentative recommendations, based on tests with several thousand seed lots, is given. Seed disinfection is recommended where the smut spore load exceeds 1:128,000 or where seed decay or seedling blight reduces the percentage of healthy seedlings from non-disinfected seed below 91%. Increases in rates of seeding are recommended when the percentage of healthy seedlings, even after seed disinfection, is less than 91% but more than 50%. Seed germinating 50% or less after disinfection is to be discarded.

Introduction

The testing of seed to measure the amount of seed-borne disease is a comparatively new and unstandardized procedure. The methods currently used include (1) the visual examination of the seed to determine its condition, (2) the examination of seed washings to determine the load of fungus spores carried on the seed, (3) the plating of the seed on nutrient agar or moist filter paper to determine the kind of fungi present within the seed, and (4) the planting of seed on some moist substrate, such as paper, peat moss, sterile sand, or sterile soil, to determine the percentage of diseased seedlings. Tests for the presence of seed-borne disease are often made in conjunction with tests of seed germinability.

While the above methods of testing seed in the laboratory for seed-borne disease possess individual merits, the common fault of most is that the seed is usually tested under conditions quite different from those under which it ordinarily germinates in the field. Often unfavourable conditions for growth, such as poor light, crowding, etc., weaken the seedlings to such a degree that they become subject to attack by relatively innocuous micro-organisms.

¹ Manuscript received July 31, 1942.

Contribution No. 719 from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Canada.

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Besides, owing to the absence of competition from saprophytes in some tests, the pathogenicity of weak pathogens may become exaggerated. In plating tests, too, it is usually impossible to separate the pathogenic from the non-pathogenic strains of the same fungal species. To overcome these difficulties, the writers considered using other methods of testing seed for the presence of seed-borne disease, and came to the conclusion that the use of non-sterile soil as a seed-testing medium offered considerable promise.

A review of the available literature yielded very little information regarding the use of non-sterile soil for seed-testing purposes. Flats or beds of non-sterile soil have been frequently used by investigators in connection with tests of seed disinfectants, but relatively seldom for tests of seed-borne disease. The general impression gained from the literature was that non-sterile soil harboured phytopathogens in dangerous quantities, and thus rendered such soil unfit for seed tests. Crosier and Patrick (3), for instance, opposed germination tests in soil, their data suggesting that the results from such soil were an index of the soil condition rather than of seed vitality, particularly if the soil did not have a low "inoculum potential."

The evidence from field experiments, however, suggests that non-sterile soil is not always a breeding ground for plant diseases. With cereals, conditions adverse to plant growth, such as high soil salinity (7), low soil moisture (6, 13), soil acidity (13), lack of nutrients (13), appear to intensify the attacks by root rotting, soil-borne fungi. In addition, recent unpublished observations by the writers showed that deep seeding also increased the severity of root rot. On the other hand, disease-free seed, when sown thinly and not too deeply in good soil, produced vigorous plants seldom damaged by soil-borne, root rotting fungi. However, under the favourable conditions just mentioned, seed infected with either *Helminthosporium sativum* P. K. & B. or *Fusarium culmorum* (W. G. Sm.) Sacc. either failed to germinate or produced weak plants that were lesioned at the base.

In view of the fact that under certain favourable field conditions cereal plants remained free from soil-borne infection, it seemed to the writers that there was much to be learned about the behaviour of non-sterile soil under greenhouse conditions. On this account, most of the experimental work described here, and carried on during the years 1939, 1940, and 1941, was devoted to a study of the conditions that would permit an attack of cereal seedlings by seed-borne fungi but would not permit an attack by soil-borne fungi. To conserve space, many of the experimental details are omitted. A part of the investigation was devoted to a comparison of the results from seed tests in non-sterile soil with those obtained by other methods.

Development of the Non-Sterile Soil Test

Preparation of the Seed Beds

Wooden frames, made of 2-in. by 6-in. planks, were set upon greenhouse benches. Only the centrally located benches were utilized because it was thought that the lateral benches, heated below by steam pipes, would make

difficult the controlling of soil temperature. Convenient widths for the seed beds were found to be 30 or 60 in., the narrower ones for small-seeded crops, such as flax, and the wider ones for cereals. The frames were filled with artificially prepared, friable soil.

A simple test of friability for unknown soils was devised. In the test, the unknown soil, air-dry and sifted, was mixed with fine sand in various proportions and the mixtures were put separately into 4½-in. ordinary earthenware pots. The filled pots were set in pans of water and left there until moisture had reached the surface of the soil. They were then removed from the pans and placed upon a greenhouse bench to drain for about an hour. When the excess water had drained out, the pots were inverted upon a flat surface and lifted from the conic frusta of moist soil within. Two or three days later, when these frusta of soil had become dry, a light, flat-bottomed dish was placed upon the upper plane of each and sand was poured into the dish until the mould of soil beneath collapsed from the weight. A mixture of soil and sand collapsing under a weight of 3 to 4 lb. was found to be friable to the desired degree. With sandy soils, lacking in organic matter, the soil was usually too porous at this degree of friability, and, with peaty soils, the soil was too retentive of moisture. Such extreme soils were not used when large scale tests with seeds were made.

After the proper mix was determined for a soil, enough of the mixture was prepared to fill the required number of seed beds. In filling the frames, a basal, 4-in. layer was put on the bottom. This layer was levelled and soaked with water, and covered with a layer of prepared soil ½ in. thick. This second layer was then levelled and furrowed transversely across the bed by pressing a bevel-edged board into it at 2-in. intervals to the depth of ½ in. Seed was planted in these furrows, covered with another ½ in. layer of prepared soil, and the finished seed bed was given a second, lighter watering.

The method of watering the seed beds was found to have a marked effect upon the uniformity of stand in the seedlings. When the thorough watering of the basal layer was omitted and the seed bed was watered only after the seed was planted and covered, the stand of seedlings was usually uneven and "spotty." On the other hand, with the watering of the basal layer, the stand was always very uniform. Also, different crops were found to require different methods of watering. With cereals, the method just described proved to be satisfactory, but with dicotyledonous crops, such as flax and certain small-seeded vegetables, the seedlings experienced difficulty in penetrating the crust of soil formed after a surface watering. To prevent a crust from forming until after the seedlings had emerged and to conserve moisture, the seed beds were covered with sheets of paper after they were sown. Following the emergence of the seedlings, the paper was removed and the soil then watered as needed.

Preparation of the Seed for Planting

Healthy seed, as well as seed damaged by fungal infection, mechanical injury, frost, and sprouting was used in the experiments described in this paper. This seed, collected from farmers in various parts of Canada, was subjected to a preliminary cleaning and then stored in tin boxes. When required in an experiment, 100-kernel lots of seed—the number of lots depending on the experiment—were counted out from the cleaned seed and planted in a seed bed, one 100-kernel lot to a row.

In experiments where disinfected as well as non-disinfected seed was used, two 100-kernel lots were counted out from each seed lot and placed separately in small coin envelopes. One of these 100-kernel lots was mixed with a dust disinfectant, applied to the seed in excess to ensure complete coverage of the seed, the surplus dust being removed by shaking the mixture in a small, wire strainer. For example, a dust disinfectant, useful for experimental purposes, was made by mixing one volume of Ceresan (5% ethyl mercury phosphate) with two volumes of powdered talc. At this dilution Ceresan did not reduce the growth of seedlings, but it prevented their infection by seed-borne or soil-borne root rotting fungi. Lower dilutions tended to stunt the seedlings, while higher dilutions gave less control of root rotting fungi. Other mercurial dusts in suitable dilutions also gave satisfactory results.

Factors Influencing the Results from Seed Beds of Non-sterile Soil

During the course of preliminary experimentation, it was found that at least three different factors, (1) soil moisture, (2) soil temperature, and (3) the time interval between the planting of seed and the collection of data, directly or indirectly influenced the results obtained from beds of non-sterile soil. As already mentioned, the method of watering influenced the uniformity of stand in the seed beds. It also affected the amount of basal lesioning caused by soil-borne pathogens in the seedlings. When the soil was kept too moist, the seedlings were frequently attacked by species of *Pythium*, and "damped-off." This form of damage occurred most often in peaty soil. On the other hand, when the soil was kept too dry, the seedlings were occasionally attacked by *Rhizoctonia Solani* Kühn. This latter observation is in accord with the findings of Hynes (6) concerning wheat and oats, and with those of Sanford (12) concerning potatoes, but it is opposed to the findings of Abdel-Salam (1), Alexander *et al.* (2), and Gratz (4) concerning vegetables.

To ascertain the most suitable conditions of soil moisture, a large seed bed of non-sterile soil was divided into three sections, the basal layer in each was watered copiously and then the sections were sown with disease-free seed of wheat, oats, and barley. After seeding, each section was watered in a different way. One of the sections received no further watering, the second section received a light watering immediately after seeding, and the third section was watered whenever the surface of the soil appeared to be dry. At the end of 10 days, when the plants were dug up for examination, the soil in the first section was quite dry, in the second section it was dry only at the surface,

while in the third section it was moist throughout. The results from four replicates of the test, with four relatively disease-free seed lots each of wheat, oats, and barley are summarized in Table I. These results show that, with a decrease in the amount of water given a seed bed, there was an increase in the percentage of blighted seedlings in wheat and barley, while there appeared to be no effect on oats. The amount of disease, however, was not very great, even in the driest seed bed. The results suggested that soil-borne disease developed least in moderately moist soil, and therefore the seed beds in later experiments were kept in this condition continually.

TABLE I

EFFECT OF THREE METHODS OF WATERING UPON THE PERCENTAGE OF SEEDLINGS WITH BASAL LESIONS FROM DISINFECTED AND NON-DISINFECTED SEED OF WHEAT, OATS, AND BARLEY

Method of watering	Wheat		Oats		Barley		Mean
	Disin-fected	Non-disin-fected	Disin-fected	Non-disin-fected	Disin-fected	Non-disin-fected	
Soil kept moist	0.36	4.46	0.12	2.44	0.76	1.45	1.59
Soil watered once	0.35	5.63	0.25	1.71	0.63	3.45	2.00
Soil not watered after seed planting	2.73	5.23	0.25	2.87	4.31	5.56	3.49
Necessary difference (5% level)	2.13	2.13	2.13	2.13	2.13	2.13	0.74

Soil temperature had only a small and indirect effect on the development of soil-borne disease. When disease-free, as well as diseased (40% of kernels infected by *Helminthosporium sativum*), undisinfected seed of Pentad wheat was planted in non-sterile, and in autoclaved, prepared soil, there was an increase in seedling blight with a rise in temperature. In view of the results obtained from the experiment concerning soil moisture, it appeared that the marked loss of soil moisture, associated with high soil temperature, was the actual cause of the increase in disease. When this experiment was repeated and the soil was kept moist, it was found that soil temperature within the range 10 to 30° C. had apparently no effect on the amount of disease, for an analysis of the experimental data (summarized in Table II) showed the differences between the percentages of blighted seedlings at the different soil temperatures to be statistically insignificant. The temperature of the soil, however, had a marked effect on seedling growth, the rate of growth increasing with the temperature. A temperature of 20° C. was found to be the most suitable for general use, for, at that temperature, the growth of seedlings was rapid, and the rate of evaporation of moisture from the soil was relatively slow.

TABLE II

EFFECT OF SOIL TEMPERATURE ON THE PREVALENCE OF BLIGHT IN THE SEEDLINGS FROM DISEASE-FREE AND FROM DISEASED PENTAD WHEAT PLANTED IN NON-STERILE AND IN AUTOCLAVED SOIL (MEANS OF FOUR REPLICATES)

Soil temperature	Percentage of blighted seedlings			
	Disease-free seed		Diseased seed	
	Non-sterile soil	Autoclaved soil	Non-sterile soil	Autoclaved soil
15° C.	0.3	0.4	30.5	55.2
20° C.	2.4	0.0	14.8	38.6
25° C.	4.4	2.8	19.6	45.7
30° C.	2.2	7.2	13.3	41.0
Necessary difference (5% level)	15.2	15.2	15.2	15.2

When seed beds of non-sterile soil were kept moist and held at approximately 20° C., the percentage of seedlings with basal lesions increased with the lengthening of the interval between the time when the seed was planted and when the seedlings were dug up. Usually, up to the sixth day, the amount of disease was small, but from the sixth day to the 12th day the increase in disease was quite rapid. On the 12th day, the percentage of seedlings with basal lesions equalled, or slightly exceeded, the percentage of wheat or barley kernels found infected, by the plating-out method, with *Helminthosporium sativum*, or, in the case of oats, with *H. Avenae* Eidam. From the 12th to the 20th day, there was only a slight increase in basal lesioning, but after the 20th day, a second rapid increase in disease occurred. The results of an experiment showing this trend in the development of basal lesioning are summarized in Table III.

TABLE III

AGE OF PLANT IN RELATION TO THE PERCENTAGE OF SEEDLINGS WITH BASAL LESIONS IN THREE LOTS OF PRATLAND BARLEY, INFECTED WITH DIFFERENT AMOUNTS OF *Helminthosporium sativum*

Age of plants (days)	Percentage of seedlings with basal lesions		
	Uninfected seed	Seeds infected, 16%	Seeds infected, 47%
8	1.2	10.5	47.7
10	2.0	8.5	56.5
12	6.0	9.5	48.6
14	3.0	15.5	60.0
16	4.7	15.2	57.0
18	6.5	16.5	59.5
20	8.5	14.7	69.2
22	7.7	25.2	75.3
Necessary difference (5% level)	5.73	5.73	5.73

The Effect of Soil Amendment or Soil Renewal

When sterile soil is used for seed beds, it is usually necessary to refill the seed beds with sterile soil for each planting. With non-sterile soil, the original soil may be left in the seed beds after the plants are dug up or it may be replaced with fresh non-sterile soil. Leaving the soil in the seed beds involves the least expenditure of labour, but since the repeated use of the same soil may result in a deterioration in its quality, an occasional changing of the soil may be necessary. The experiment described below indicates that, with tests of cereal seed, such changes need not be frequent.

A large bed of non-sterile soil, the soil of which had been used continuously for a period of six months, was divided by means of boards into four equal sections. The soil in one section was replaced with "new" soil of the same kind. To the soil in the second section, ammonium phosphate fertilizer (11-48) was added at the rate of 11 gm. to two Imperial gallons of soil. The soil in the third section was watered with a solution of thiamin hydrochloride (vitamin B₁) in the concentration recommended by the manufacturer (15 International Units in 1 gal. of water) instead of with water alone. The soil in the fourth section was left unchanged and unamended. In each section, two lots each of wheat, oats, and barley were sown, and the seed beds were kept moist and at about 20° C. Ten days after seeding, the seedlings were lifted, counted, examined for the presence of basal lesions, and weighed while green. The results of this experiment showing the averages for each section of the bed (four successive plantings in a section) are shown in Table IV. These results indicate that the addition of soil amendment or changing the soil influenced neither the percentage of seeds germinating nor the green weight. The failure to affect the green weight showed that the soil had not become seriously depleted of nutrients even after six months of continuous use. There was, however, a significant difference between the mean percentages of plants with basal lesions, that is, more seedlings were infected in the "new" soil than in the "old" soil.

TABLE IV

THE INFLUENCE OF SOIL AMENDMENT AND SOIL RENEWAL ON THE PERCENTAGE OF SEEDS GERMINATING, THE PERCENTAGE OF SEEDLINGS WITH BASAL LESIONS, AND THE TOTAL GREEN WEIGHT OF SEEDLINGS FROM 100 SEEDS

Factor studied	Unamended "old" soil	Fertilizer added	Vitamin B ₁ added	"New" soil	Necessary difference (5% level)
Germination	94.2	94.6	94.5	93.6	1.06
Seedlings with basal lesions	0.4	0.8	0.7	1.9	0.55
Green weight (gm.)	22.7	22.1	22.5	21.7	1.21

Amount of Variability Between Different Seed Beds and Plantings

Beds of non-sterile soil, prepared, planted, and maintained in the way already described, showed very little variability in the results obtained from them. For instance, when a seed lot of disease-free wheat was planted in each of seven different seed beds located in four different greenhouse sections, and replicate plantings were made one, two, and three months after the initial planting, the variability in germination and disease was small, and the differences were statistically insignificant. In each instance, germination was over 90%, the range between the maximum and minimum percentages being seven per cent for disinfected seed and nine per cent for non-disinfected seed. The range in the percentage of seedlings with basal lesions was even less, being only one per cent in the plants from disinfected seed and four per cent in the plants from non-disinfected seed. In another trial, with 12 lots of wheat seed differing in amount of disease, the differences between seed beds and plantings were also statistically insignificant. It was assumed, therefore, that if the seed beds were maintained in the proper condition, the results of the non-sterile soil test were unlikely to be affected by minor differences between greenhouses.

The Collection of Data from Plantings in Beds of Non-sterile Soil

After trials with several hundred different lots of seed of wheat, oats, and barley that had been planted in beds of non-sterile soil, it was concluded that by following a relatively simple procedure a great deal of information could be obtained for any seed lot. Firstly, by examining the rows of seedlings growing in the seed beds, it was often possible to identify the varieties of wheat in lots of mixed seed, or the variety when the varietal name of the seed was not given. For instance, the varieties Thatcher, Renown, and Regent, which embrace the bulk of the wheat crop in Manitoba, were readily distinguishable. In the seedling stage, Thatcher was found to have smooth leaves and practically no reddish pigment in the coleoptile. Renown, on the other hand, was found to possess both pigment and rough leaves. Regent differed from Renown in that the pigment was generally absent, but the leaves were rough. Among the durum wheats, none of the varieties tested had rough leaves and only the variety Golden Ball showed any pigmentation. In oats and barley, characters definitely distinguishing one variety from another were not found. Secondly, the germinability of seed could be determined by counting the seedlings that had emerged from the soil. Ordinarily, because the amount of basal lesioning was used as an index of seed infection by root rotting fungi, this count of seedlings was delayed until after they were dug from the soil and cleaned. As each lot of seedlings was dug, they were divided into two groups, one containing seedlings with basal lesions and one containing seedlings that showed no lesions. Each group was then separated into three subgroups, the first containing vigorous plants, the second containing weak plants, and the third containing distorted plants. Weak plants were generally found to arise from shrunken, diseased, or broken seed, while the distorted seedlings originated from seed damaged by frost, sprouting, or, as in oats,

from seed infected with *Helminthosporium Avenae*. The percentage of healthy, vigorous seedlings was taken as an index of the seed value of any particular seed lot. Thirdly, when a row of disinfected seed as well as a row of non-disinfected seed of the same seed lot was planted, it was possible, by comparing the results from the two rows, to assess the value of seed disinfection for the particular seed lot. The test with disinfected seed was taken to show the maximum germinability of the seed. Generally, unless the seed was free from infection by root rotting fungi, seed disinfection increased the percentage of vigorous plants.

Comparison of the Results Obtained by the Non-Sterile Soil Method of Testing Seed with Those Obtained by Other Methods

When the non-sterile soil method of seed testing had been developed to a point where repeated tests with a seed lot gave relatively uniform results, this method was compared with other methods of seed testing. This comparison was made with the object of determining the relative accuracy of the different methods, as well as of determining the relative amounts of time, labour, and costs of materials consumed in following them.

The first comparison was between non-sterile soil and autoclaved soil. In Table II the data show that, with diseased wheat, fewer seedlings became diseased in non-sterile soil than in soil that had been autoclaved. As this result was obtained from soil in relatively small (1 gal.) containers, the result was not thought wholly comparable to the result obtainable from large seed beds, and therefore the experiment was repeated, but on a larger scale. To obtain comparable conditions, a large seed bed was partitioned into three sections, one of which held non-sterile prepared soil, another held prepared soil that had been autoclaved at 15 lb. pressure for three hours, and a third held a 4-in. layer of non-sterile soil on the bottom and a 1-in. layer of autoclaved soil, in which the seed was planted, at the top. In each section of soil, disinfected and non-disinfected seed from two lots each of relatively disease-free seed of wheat, oats, and barley were planted. Ten days later, the seedlings were dug, counted, and examined for the presence of basal lesions. The results (averages of four replicates) are summarized in Table V. They show that, contrary to the general belief, less disease developed in non-sterile soil than in sterile soil, while the germination of seed was equally good in both. In the two-layer soil, the amount of disease was slightly more than in the other two soils. A slightly different result was obtained from a similar experiment, but with diseased seed. In this second experiment, there was (Table VI) least disease in the two-layer soil, while the results from non-sterile soil and from sterile soil were very much alike. The results of these two experiments show, therefore, that a test of seed in non-sterile soil may be expected to give results very similar to a test of seed in sterile soil. This being the case, a test in non-sterile soil is to be preferred, as the expenditure of time and labour involved in soil sterilization is avoided.

TABLE V

EFFECT OF DIFFERENT SOIL TREATMENTS ON THE PERCENTAGE OF SEEDS GERMINATING AND THE PERCENTAGE OF SEEDLINGS WITH BASAL LESIONS IN RELATIVELY DISEASE-FREE SEED OF WHEAT, OATS, AND BARLEY

Factor studied	Seed treatment	Crop	Soil treatment		
			Non-sterile	Autoclaved	Non-sterile and autoclaved
Germination	Disinfected	Wheat	96.5	96.0	95.5
		Oats	97.5	98.0	97.5
		Barley	98.0	97.5	97.5
	Non-disinfected	Wheat	96.0	97.5	95.5
		Oats	98.0	98.0	95.5
		Barley	96.5	97.0	97.0
Mean*		97.0	97.3	96.4	
Seedlings with basal lesions	Disinfected	Wheat	0.0	0.1	0.2
		Oats	0.0	0.0	0.1
		Barley	0.0	0.0	0.3
	Non-disinfected	Wheat	1.0	1.8	1.7
		Oats	1.0	1.3	1.9
		Barley	0.8	2.1	2.7
Mean**		0.46	0.88	1.15	

* Difference between means not significant.

** Difference of 0.57, significant at 5% level.

As already mentioned, another common method of testing seed for seed-borne disease is to plate the seed out on nutrient agar. This method, while showing the degree to which the seed is infected with pathogenic as well as non-pathogenic fungi, does not indicate better than roughly seed germinability.

The data in Table VI indicate that the percentage of seeds found, by a plating test described elsewhere (8), to be infected with seedling blight fungi (*Helminthosporium sativum* and *H. Avenae*) is closely related to the percentage of seedlings with basal lesions when the diseased seed was planted in non-sterile soil. These data were obtained from tests with only a few seed lots and were not considered to be conclusive. When, however, the results of plating tests with several hundred seed lots of wheat, oats, and barley were compared with the results obtained from the same seed lots planted in non-sterile soil, the relation became more pronounced. As shown in Table VII, an increase in the percentage of seeds infected with either *H. sativum* or *H. Avenae* was accompanied by an increase in the percentage of seedlings with basal lesions. In wheat, there was a tendency for the percentage of infected seeds to be higher than the percentage of lesioned seedlings, a tendency that was reversed in oats. In barley, the percentages were nearly equal. The

TABLE VI

EFFECT OF DIFFERENT SOIL TREATMENTS ON THE PERCENTAGE OF SEEDS GERMINATING AND ON THE PERCENTAGE OF SEEDLINGS WITH BASAL LESIONS IN DISEASE-FREE AND DISEASED SEED OF WHEAT, OATS, AND BARLEY

Soil treatment	Percentage of diseased kernels in seed*														
	Wheat					Oats					Barley				
	0	0	2	24	Mean (A)	0	1	3	20	Mean (A)	2	6	21	34	Mean (A)
Percentage of seeds germinating**															
Non-sterile	97	97	95	76	91.2	97	98	98	95	97.0	94	99	96	97	96.5
Autoclaved	98	95	97	86	94.0	97	98	98	93	96.5	98	98	98	97	97.7
Two-layer	95	97	96	88	94.0	98	95	96	94	95.7	95	99	94	98	96.5
Mean (B)	96	96	96	83		97	97	97	94		95	98	96	97	
Percentage of seedlings with basal lesions***															
Non-sterile	1.0	5.0	1.0	27.8	8.7	0.2	0.5	1.5	26.5	7.2	0.0	1.0	24.2	35.0	20.0
Autoclaved	1.5	1.5	2.2	33.3	9.6	0.0	2.5	0.2	27.2	7.5	0.0	2.0	19.6	26.5	12.0
Two-layer	2.0	0.7	1.5	27.5	7.9	0.2	1.2	2.7	24.3	7.1	0.5	1.5	11.5	20.2	8.4
Mean (B)	1.5	2.4	1.5	25.6		0.1	1.4	1.4	26.0		0.2	1.5	18.4	27.3	

* Wheat and barley infected with *Helminthosporium sativum*, oats infected with *H. Avenae*.

** Difference for means (A) not significant, but for means (B) a difference of 1.71, significant at 5% level.

*** A difference of 2.01 for means (A) and a difference of 2.83 for means (B), significant at 5% level.

difference, in wheat, was thought due to an infection of some of the seed with strains of *H. sativum* incapable of causing basal lesioning in non-sterile soil under the conditions of the test. On the other hand, the difference in oats seemed due to a superficial infection by *H. Avenae* in some seed lots. This superficial infection, proved by other tests, was thought to be destroyed when the oat seed was surface sterilized prior to being plated out. The amount of infection of the seed with other pathogenic fungi, such as *H. teres* Sacc. in barley and *Fusarium* spp. in wheat, oats, and barley, apparently had no relation to the amount of basal lesioning. When the percentage of seeds infected with either *H. sativum* or *H. Avenae* was added to the percentage of seeds infected with *H. teres* or *Fusarium* spp., or both, the closeness of the relation between seed infection and basal lesioning decreased. These results were taken to mean that, of the fungi considered, only *H. sativum* and *H. Avenae* attacked the seedlings of cereal crops in non-sterile soil. Other seed-borne, seedling blight fungi, such as *Fusarium culmorum* (W. G. Sm.) Sacc. or *Gibberella Saubinetii* (Mont.) Sacc., were not encountered in any of the seed lots examined, and for this reason a comparison between a plating test

TABLE VII

THE RELATION BETWEEN THE PERCENTAGES OF SEEDS FOUND INFECTED, BY THE PLATING TEST, WITH PATHOGENIC FUNGI, AND THE PERCENTAGES OF SEEDLINGS WITH BASAL LESIONS

Fungus	Percentage of seeds infected with fungus	Average percentage of seedlings with basal lesions		
		Wheat	Barley	Oats
<i>Helminthosporium sativum</i>	0-5	1.85	2.60	13.40
	6-10	4.34	4.17	5.20
	11-20	5.28	7.70	*
	21 +	*	17.83	*
<i>Helminthosporium teres</i>	0-5	**	3.52	**
	6-10	**	0.57	**
	11-20	**	1.66	**
	21 +	**	3.66	**
<i>Helminthosporium Avenae</i>	0-5	**	**	7.42
	6-10	**	**	15.18
	11-20	**	**	24.60
	21 +	**	**	43.00
<i>Fusarium</i> spp. ***	0-5	0.92	3.30	12.56
	6-10	2.08	4.55	12.65
	11-20	0.00	2.25	10.52
	21 +	*	*	8.00

* No seed lots with this class of infection.

** Fungus not found in seed of this crop.

*** *Fusarium Poae*, *F. Avenaceum*, *F. Scirpi*.

and a non-sterile soil test for these fungi could not be made. It is thought, on the basis of previous experience with these fungi, that the relation would be similar to that already found for *H. sativum* and *H. Avenae*.

In a third comparison, the results from non-sterile soil were compared with those obtained when non-disinfected seed was germinated on moist paper in incubators maintained at constant temperature and humidity. For purposes of comparison, a group of 40 seed lots each of wheat, oats, and barley were selected from among a large number of samples collected from the grain growing areas throughout Canada. The selected seed lots represented healthy seed as well as seed that was sprouted, shrivelled, injured by frost, injured mechanically, or infected with pathogenic fungi. Each lot was divided into three parts. One of the parts was plated to determine the prevalence of infecting fungi, but particularly of *Helminthosporium sativum*, *H. teres*, and *H. Avenae*. The second part was subdivided into two, of which one was treated with diluted Ceresan and the other left untreated, and both were sown in a bed of non-sterile soil. The third part (non-disinfected) was germinated upon moist paper (paper towels) in the incubator at 75° F. and in a saturated atmosphere¹. The results of the three tests were then summarized and correlated, as shown in Table VIII.

¹ This part of the experiment was performed in the laboratory of the North West Line Elevators Association, Winnipeg, Man., through the courtesy of Dr. K. W. Neatby, Director of the Agricultural Department.

TABLE VIII

COMPARISON OF THE PERCENTAGES OF SEEDS GERMINATING AND THE PERCENTAGES OF DISEASED SEEDS OR SEEDLINGS OBTAINED WITH WHEAT, OATS, AND BARLEY WHEN TESTED BY THREE DIFFERENT METHODS OF TESTING SEED

Factor studied	Kind of test	Seed treatment	Crop		
			Wheat	Oats	Barley
Percentage of seeds germinating	Soil (a)	Treated**	94.60	93.03	93.52
	Soil (b)	None	89.52	87.24	93.46
	Moist paper (c)	None	92.54	92.48	93.81
Percentage of diseased seeds or seedlings	Plating-out (d)	Treated***	3.16	4.94	8.20
	Soil (e)	None	3.49	15.99	7.53
	Moist paper (f)	None	38.43	2.07	8.17
Coefficients of correlation	Between (a) and (b)		0.566*	0.897*	0.891*
	Between (b) and (c)		0.545*	0.750*	0.665*
	Between (d) and (e)		0.628*	0.020	0.720*
	Between (d) and (f)		0.238	0.003	0.372*
	Between (e) and (f)		0.309*	0.215	0.363*

* Denotes attainment of 1% level of significance.

** Dusted with dilute New Improved Ceresan.

*** Disinfected with mercuric-chloride-alcohol solution before being plated out.

With respect to the percentage of seeds germinating, this percentage was highest when the seed was dusted with dilute Ceresan and then planted in non-sterile soil. The percentage was lowest when non-dusted seed was planted in non-sterile soil. The coefficients of correlation for the percentages of seeds germinating show that germination on moist paper was more comparable to a soil test with disinfected seed than to a soil test with non-disinfected seed.

The percentage of infected seed or seedlings was found to vary with both the crop and the test. In wheat, the percentage of seeds found by the plating test to be infected with pathogenic fungi agreed closely with the percentage of lesioned seedlings from non-disinfected seed in non-sterile soil. The coefficient of correlation for these two tests was quite high (0.628). On moist paper, however, a large number of the wheat seedlings became infected secondarily through a spread of pathogenic fungi from infected seeds. On this account, the coefficient of correlation between the results of this test and those of the plating test was very low. The correlation between the moist paper test and the non-sterile soil test was also very low. In oats, the percentage of seedlings developing disease was greatest in non-sterile soil and least on moist paper. The percentage of seedlings showing disease in non-sterile soil was considered to be a more reliable index of the amount of seed infection by *Helminthosporium Avenae* than was the percentage of seeds found infected by the plating test. When the seed was planted in soil, practically every seedling found diseased showed a lesion typically produced by *H. Avenae*.

As this fungus apparently does not grow in non-sterile soil (10), the lesions evidently originated from infected seed. In contrast, the seedlings on moist paper failed to produce lesions typical of the disease. When the results of the three tests with oats were correlated, none of the coefficients of correlation were significant. In barley, as in wheat, there was close agreement between the results of the plating test and the test in non-sterile soil, while there was relatively little agreement of the results from either of these tests with those obtained on moist paper.

With respect to the different kinds of physical damage to the seed, experience showed that the test in non-sterile soil measured the amount of damage more accurately than did a test in plates of nutrient agar or a test on moist paper. Sprouted seed germinated better in soil than it did on moist paper. Many of the sprouted-seed lots, when dusted with dilute Ceresan and planted in soil, germinated surprisingly well. Shrivelled seed germinated as well on moist paper as in soil, but the difference between the diameters of the coleoptiles from shrivelled kernels and those from plump kernels was more pronounced in soil than on moist paper. The effect of frost injury to the seed was much more evident in the soil test than in the moist paper test. On moist paper, only severe frost injury produced any noticeable effect, while, in the soil test, even a slight degree of injury was readily evident. In soil, slight damage by frost to the seed resulted in a characteristic waviness of the veins near the tip of the primary leaf. With moderate damage, the entire primary leaf, sometimes also the second and third leaves, showed this waviness. With severe damage, the seed either failed to germinate or produced a crinkled, stunted plant.

Experiments with Barley Seed Infected with *Helminthosporium teres*

When barley seed, collected from various parts of Canada during the years 1938-1941, inclusive, was subjected to a plating test, it was found that infection of the seed with *Helminthosporium teres* was quite prevalent, the range of infection varying from 1 to 64%. However, when the infected seed was planted in beds of non-sterile soil, there was seldom any evidence of the disease in the seedlings. Severely infected seed lots produced a few plants with leaf lesions, but there was scarcely any basal lesioning. When infected seed was germinated at room temperature on moist paper in Petri dishes, spores of *H. teres* formed on the surface of the seed but no lesions developed on either the stems or leaves.

In view of the results obtained by Ravn (11), it was thought that the failure of the lesioning to appear was due to the relatively high temperature (20° C.) at which the seed was germinated in the soil. On this account, several modifications of the non-sterile soil method of testing seed were tried. In the first experiment, three lots of infected barley seed were planted in pots of non-sterile soil, the pots then being placed in a cool greenhouse (10° to 12.5° C.). They were kept at this temperature until the seedlings had

begun to emerge from the soil (eight days after planting). At this time, one-third of the pots were removed to a greenhouse kept at 20° C. Later, the remaining pots were removed to the warmer greenhouse, one-half of the remainder at the end of two days and the rest at the end of four days. The plants were examined when the third leaf had begun to show in the majority of plants in each set of pots. The results from four replicates of this test showed no difference whatever in the amount of leaf lesioning between the three sets of pots. There was a difference in the amount of leaf lesioning between seed lots, however, for two of the lots of seed (with 18% and 34% infection, respectively—determined by a plating test) showed only a trace of lesioning, while the third lot, with 49% of the seeds infected, produced 14% of plants with leaf lesions. There was scarcely any difference at all between sets of pots and between seed lots in the percentage of plants with basal lesions.

In a second experiment, the three seed lots just referred to were planted in autoclaved and in non-sterile soil, but otherwise the experimental procedure was the same as in the previous experiment. The results from four replicates of this experiment showed that there was very little difference in the amount of leaf lesioning produced in autoclaved and in non-sterile soil (10% and 11%, respectively). There was a significant difference, however, in the amount of basal lesioning. In autoclaved soil, 13.6% of the plants showed basal lesions while in non-sterile soil only 5.6% were thus affected.

In a third experiment, only one seed lot (49% infection with *H. teres*) was used, but the period of incubation in the cool greenhouse (10° to 12.5° C.) varied from 0 to 24 days. The seed was planted in quadruplicate sets of pots of non-sterile prepared soil to furnish four replicates of the test. Four pots from each set were placed in a greenhouse at 20° C., while the remaining pots were placed in the cool greenhouse mentioned above. At daily intervals afterwards until the seventh day, a group of 16 pots was removed to the warmer greenhouse, but one group of 16 pots was left in the cool greenhouse for 24 days. The plants were examined for leaf and basal lesions when the third leaf appeared. The results of the examination are summarized in Table IX. They show that, after the fourth day, there was a progressive increase in leaf lesioning with an increase in length of the incubation period at low temperature, and that there was no increase or decrease in the amount of basal lesioning.

In a fourth experiment with *Helminthosporium teres* a number of different lots of barley seed were planted in a large bed of non-sterile soil. The temperature of the greenhouse was kept at 10° to 12.5° C. for seven days after the seed was planted, and then raised to 20° C., at which temperature it was held for seven days. The percentages of seedlings with leaf lesions were determined at the end of the second seven day interval. Three further tests were made with the same seed and under the same conditions. The summarized results are given in Table X, and they show that the percentage of seedlings with leaf lesions is not necessarily related to the percentage of seeds

TABLE IX

RELATION OF DIFFERENT PERIODS OF INCUBATION AT 10° TO 12.5° C.
ON THE PERCENTAGES OF SEEDLINGS WITH LEAF AND BASAL
LESIONS IN A LOT OF BARLEY SEED SEVERELY INFECTED
WITH *Helminthosporium teres*

Incubation period (days)	Percentage of seedlings with leaf lesions	Percentage of seedlings with basal lesions
0	2.25	3.50
1	3.00	4.50
2	5.00	4.25
3	2.75	2.50
4	6.00	1.50
5	9.75	2.25
6	12.75	3.00
7	15.25	3.50
24	20.50	2.00
Necessary difference (5% level)	4.80	3.52

that are infected. This seems to indicate that different seed lots of barley may be infected with different strains of *H. teres*. The difference between seed lots Nos. 14 and 15 is striking enough to suggest this possibility. Under such circumstances, a plating test with seed is obviously misleading. The experience of the writers, based on tests with hundreds of seed lots of barley, indicates that the proportion of barley seed lots infected with virulent strains of *H. teres* is relatively small.

A field experiment with infected barley seed was conducted in 1940. In this experiment, eight lots of barley seed, with an infection range from 0 to 34% were planted in rod rows on May 8 and the seedlings were examined one month later. The results were in accord with the greenhouse results shown in Table X. Only a trace of leaf lesioning appeared in the seed infection range from 0% to 15%, while in a seed lot with 34% of the seeds infected, 4.3% of the seedlings bore the reticulate lesions typically caused by the fungus. A second field experiment, made in 1941, gave a different result. In this, the percentages of seedlings with leaf lesions in the field agreed closely with the percentages of seeds found infected by the plating test, but when the same seed lots were planted in non-sterile soil under greenhouse conditions (20° C.), only one lot, with 25% of the seed infected, produced seedlings with lesions on the leaves.

These results of greenhouse and field experiments with barley seed indicate a variability in the pathogenicity of *Helminthosporium teres*, and also a relative unreliability of the non-sterile soil test for the appraisal of the amount of seed infection in barley by *H. teres*. There are indications, also, that the plating test for this fungus is also unreliable, as it apparently does not differentiate between virulent and non-virulent strains of *H. teres*. Tentatively,

TABLE X

RELATION OF THE PERCENTAGE OF SEEDS INFECTED WITH *Helminthosporium teres* TO THE PERCENTAGE OF SEEDLINGS WITH LEAF LESIONS WHEN THE SEEDLINGS WERE FIRST GROWN FOR SEVEN DAYS AT 10° TO 12.5° C. AND FOR AN ADDITIONAL SEVEN DAYS AT 20° C.

Seed lot number	Percentage of seeds infected with <i>H. teres</i>	Percentage of seedlings with leaf lesions
1	0	0.50
2	1	0.50
3	2	0.25
4	4	0.25
5	5	1.00
6	6	0.75
7	7	0.50
8	11	1.00
9	12	0.00
10	18	0.00
11	19	4.25
12	23	0.50
13	34	0.50
14	38	0.00
15	49	17.75
Necessary difference (5% level)		1.50

however, the plating test may be said to be the more accurate, because it does show when the fungus, whether innocuous or not, is present.

Recommendations Based on the Test in Non-Sterile Soil

Two distinct quantities have been measured by the non-sterile soil test for every sample that was tested, namely, (1) the percentage of seeds that was viable, and (2) the percentage of seeds producing healthy plants. From the farmer's view-point, the determination of the latter is probably the more important.

Following an examination for disease of several thousand samples of cereal seed (wheat, oats, and barley) in 1938, 1939, 1940, and 1941, it became evident that the samples could be classified into two principal groups, namely, samples in which the seed was free from smut or contained only a trace, and samples containing more than a trace of smut (covered smuts of wheat, oats, and barley, and loose smut of oats). The degree of infection by smut was determined by centrifuging the washings from the seed (5). These two principal groups of samples could be subdivided into two subgroups, namely, samples that germinated well either without or after seed treatment and samples that germinated poorly even after treatment. A further subdivision could be made on the basis of the degree to which seed disinfection increased the percentage of healthy plants. Within the main groups and subgroups just mentioned, there were several minor groups including (1) frosted seed, (2)

seed in which treatment increased the sturdiness of the seedlings without increasing their number, and (3) seed producing weak seedlings even after treatment.

On the basis of this grouping of seed samples, it was possible to set up a table of tentative recommendations regarding the handling of seed represented by the samples tested in non-sterile soil. These recommendations (Table XI) are based partly upon the results from unpublished field experiments made by the present writers and partly upon the information regarding smuts obtained from Mr. W. Popp, of this laboratory.

TABLE XI

RECOMMENDATIONS, BASED ON A CENTRIFUGE TEST FOR SMUT AND A SOIL TEST FOR PHYSICAL AND FUNGUS INJURY, REGARDING THE HANDLING OF CEREAL SEED BEFORE SEEDING

Smut spore load on seed	Percentage of healthy plants			Recommendations
	Treated seed	Non-treated seed	Increase from treatment	
Heavy trace to heavy††	91-100	*	*	Treat seed with organic mercury dust Treat seed and increase rate of seeding** Discard seed See footnote***
	51-90	*	*	
	0-50	*	*	
	Frosted seed	*	*	
None or trace	91-100	91-100	0-9	No treatment required Treat seed with organic mercury dust Increase rate of seeding** Treat seed and increase rate of seeding Discard seed See footnote***
	91-100	0-90†	5 +	
	51-90	51-90	0-5	
	51-90	51-85	5 +	
	0-50	—	—	
	Frosted seed	—	—	

* On account of infection with smut, all seed should be treated.

** Percentage increase in rate of seeding = $\frac{(100-x)}{x} \times 100$ where x = % of healthy plants in treated seed.

*** Ordinarily about 50% of frosted seeds produce normally growing plants. Theoretical percentage of healthy plants = % healthy seedlings + $\frac{\% \text{ frosted seedlings}}{2}$. Calculate increased seeding rate on basis of theoretical percentage.

† In barley, subtract percentage of seeds found by plating test to be infected with *Helminthosporium teres* from percentage of healthy seedlings from untreated seed.

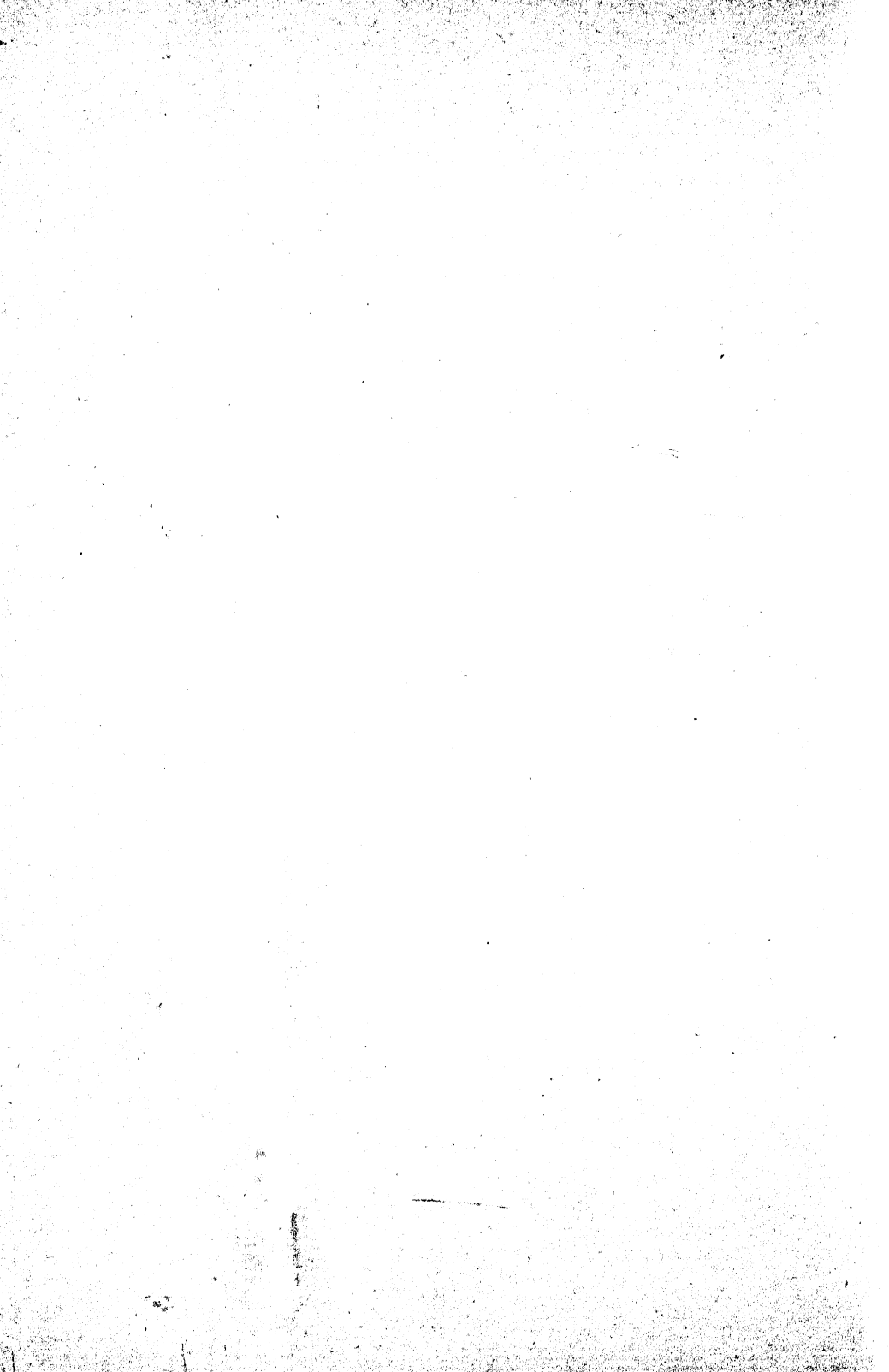
†† Smut spore load exceeding 1 : 128,000.

It is difficult to formulate recommendations equally applicable to areas with different climatic conditions. The results of field experiments (9) have shown, for instance, that in an area with semiarid climatic conditions, a low rate of seeding is likely to give a yield equal to that from a high rate of seeding, and have shown that, from seed treatment, moderate increases in density of stand fail to give any increase in yield. In such areas, therefore, low germinability of the seed, or the infection of the seed by seed-borne seedling blight fungi, seems to be of less consequence than in areas where the soil moisture is sufficient to support a dense population of plants. Similarly, the

writers found that, with seed-borne leaf-spotting diseases, infection may spread from the seed to the seedlings, but the relatively dry weather of summer, for example in Manitoba, prevents the disease from spreading in the field and doing harm. Under humid weather conditions, however, these diseases increase in destructiveness with the progress of the season. Thus, a high degree of seed infection by leaf spotting fungi may not reduce yields when the seed is sown under semiarid conditions, while even a trace of infection may be destructive when the seed is sown under humid conditions. Evidently, from these observations, seed of relatively low quality may produce a good crop in semiarid areas, while seed of only the best quality can do so in humid areas. This, however, should not be interpreted as suggesting that there should be different standards of seed quality for different areas, but only that, under certain conditions, seed with moderate germinability and a moderate amount of seed-borne disease may give yields equal to those from the best quality seed.

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Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 20, SEC. C.

DECEMBER, 1942

NUMBER 12

EXPERIMENTS ON CHEMICAL CONTROL OF DAMPING-OFF IN *PINUS RESINOSA* AIT.¹

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Abstract

Results from a series of greenhouse and nursery experiments on the relative control of damping-off in red pine, afforded by a wide range of chemicals, brought out the following points: (1) Semesan solutions in concentrations of 1 : 100 to 1 : 150 applied as soil treatment gave consistently the best control in both greenhouse and nursery. (2) Red copper oxide suspension in concentrations of 1 : 250 to 1 : 500 applied as a soil treatment was also effective, particularly in the greenhouse. (3) Red copper oxide and zinc oxide dusts used as seed treatments gave effective control under greenhouse conditions. (4) In the greenhouse, combinations of seed and soil treatments failed to prove more effective than treatments applied separately, while under certain nursery conditions the combined treatments were significantly less effective. (5) Fungicides as a group generally proved more effective than acidifying agents such as sulphuric acid and aluminium sulphate. It appeared that the acidifying agents gave good control in seasons of normal rainfall, but were more or less ineffective in dry seasons, when relatively large quantities of slightly alkaline irrigation water were applied, or in wet seasons, when excessive percolation occurred. (6) Seedlings from two-year-old red pine seed of somewhat reduced vitality proved to be much more susceptible than those from one-year-old seed.

White spruce was used in some of the preliminary experiments and proved to be much less susceptible than red pine, although very similar in response to the various treatments.

Introduction

Seedlings of several species of forest trees are subject to damping-off attacks prior to and for a few weeks after emergence from soil. Recurrent losses from this disease, particularly in red pine, coupled with an apparently inadequate existing knowledge of means of control, led the authors to undertake the greenhouse and nursery experiments reported herein.

The greenhouse studies, extending over the winters of 1939-40 and 1940-41 involved a series of experiments carried out in the following sequence: preliminary studies on chemical treatments, both seed and soil, conducted under different conditions of temperature and humidity, using both red pine and white spruce; exploratory tests with red pine in which 15 chemicals,

¹ Manuscript received October 9, 1942.

Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, and the Division of Reforestation, Department of Lands and Forests, Toronto, Ont. A part of a co-operative project of the Subcommittee on Forest Tree Breeding, Associate Committee on Forestry. N.R.C. No. 1090.

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mostly fungicides, were used at various concentrations and applied in different ways, singly and in combinations; and, combination seed and soil treatments, based on results of exploratory tests, tested on red pine in a factorial experiment.

A nursery experiment of factorial design involving combination seed and soil treatments based on greenhouse results was duplicated at Ottawa and Orono in 1941. Extensive experiments on soil acidification were also carried out at the Orono nursery during the period 1939-1942.

Because of the fact that each succeeding test was based on the preceding one, it has been considered sufficient to present details of only the later experiments. The results of most of the earlier work are discussed but not tabulated.

Literature reviews on the subject of damping-off control have been recently published by Boyce (1) and Horsfall (3).

Materials and General Methods

Red or Norway pine, *Pinus resinosa* Ait., was used throughout the tests; *Picea glauca* (Moench) Voss. was also used in some of the earlier experiments.

The chemicals tested included the fungicides, Semesan, Semesan Jr., mercuric chloride, ethyl mercuric bromide, red copper oxide, black copper oxide, copper carbonate, copper sulphate, zinc oxide, Du Bay, and Cheshunt compound, and the acidifying agents sulphuric acid, acetic acid, and aluminium sulphate. For the most part these chemicals have been recommended in the literature as being effective in reducing damping-off losses, especially in relation to vegetable garden plants.

The fungicide Semesan contains 30% hydroxymercurichlorophenol, the mercury equivalent in different lots used varying from 17.4 to 19.0%. The "New Improved" type of Semesan Jr. was used, which contains one per cent ethyl mercury phosphate. The preparation referred to as Du Bay is manufactured by the E. I. du Pont de Nemours and Co. Inc., Wilmington, Delaware, under the number 1155-HH. Cheshunt compound is a "home" preparation which contains 15.4% copper sulphate and 84.6% ammonium carbonate by weight.

The standard laboratory chemicals used were all of technical or commercial grade.

In the greenhouse experiments, water-tight crocks 6½ in. in diameter (inside) and 9 in. in height were used as soil containers. Each crock was brought up to equal tare weight and 4.5 kg. of soil of known moisture content added. This permitted maintenance of soil moisture content at approximately 20% by means of periodic weighing of the crocks and the addition of the amount of water necessary to bring them up to the required weight.

In steam sterilization, the soil was subjected to two hours treatment with live steam at 15 to 18 lb. pressure.

All tests were designed with either two or three randomized replicates.

Dosage for dust treatments was in all experiments the maximum amount adhering to the surface of dry seed.

Rates of seeding in the greenhouse varied with germinability of the seed used. The criterion was to sow the number of seeds which, on the basis of a prior germination test, would be expected to produce about 100 seedlings per crock. The white spruce seeds were stratified for five weeks at 36° F.

Greenhouse temperatures were controlled within one degree Fahrenheit—except during periods of particularly strong sunlight. Humidity was less accurately controlled by means of moist peat on and under the benches. Hydrothermographic records were kept during all experiments.

Seedling counts were made at the end of the fourth week after sowing and continued for five weeks thereafter. Total seedlings and number dampened-off were counted each week, the latter being removed to facilitate future counting. Records were kept in a cumulative manner. Only the data of the final count were used in the analyses.

Data on pre-emergence damping-off were obtained indirectly: instances where the total number of emerged seedlings in treatment crocks significantly exceeded the number in corresponding check crocks were taken to indicate a degree of control of pre-emergence damping-off.

Since the terms "pre-emergence damping-off" and "postemergence damping-off" are very inconvenient to use and since the latter is the one commonly dealt with, the term "damping-off" is used, unless otherwise noted, to refer to the postemergence form.

Experimental Results (Greenhouse)

PRELIMINARY EXPERIMENTS

1. *Relation of Temperature and Humidity to Damping-off*

Three greenhouse sections were maintained under more or less constant temperature and humidity conditions, as follows:

55° F. and 40 to 55% relative humidity,

70° F. and 25 to 35% relative humidity,

70° F. and 45 to 65% relative humidity.

The experiment involved a total of 216 crocks of contaminated soil sown in equal proportions to red pine and white spruce. Both seed and soil treatments were tested in replicate (the more pertinent results from which are included in Section 2 below).

At the lower temperature (55° F.), only a low percentage of damping-off occurred, but germination was very poor in red pine and only fair in white spruce.

At 70° F., the higher humidity (45 to 65%) significantly increased the number of seedlings emerged, and later significantly increased the percentage damping-off among emerged seedlings. The means by which the higher humidity produced increased emergence, whether as a beneficial effect on germination or as a reduction in pre-emergence damping-off, is not known. In any case, it was clear that the experimental conditions obtained at 70° F.

and the higher humidity were the most desirable for testing damping-off control, and hence were chosen for all subsequent greenhouse experiments.

2. Tests of Seed and Soil Treatments

The main preliminary experiment involved the testing of 15 chemicals which, in consequence of some being used as both seed and soil treatments or in more than one concentration, formed the basis of 41 different tests. Each test was replicated three times in Ottawa soil (inoculated with Orono soil) and three times in Orono soil (from beds heavily infested with damping-off organisms) making, with replicated steam sterilization treatment and checks, a total of 258 crocks. Only red pine seed was used.

The chemicals were applied as follows:

Liquid seed treatments—

Copper sulphate, 30 min. soaking in 1 : 80 solution.

Formalin, 60 min. soaking in 1 : 300 solution.

Dust seed treatments—

Semesan, Semesan Jr., red copper oxide, black copper oxide, zinc oxide, copper carbonate.

Liquid soil treatments—

Semesan (1 : 400, 1 : 200), Semesan Jr. (1 : 5000, 1 : 1000), sulphuric acid (1 : 200, 1 : 100, by volume), formalin (1 : 300, 1 : 150, by volume), aluminium sulphate (1 : 40, 1 : 20), mercuric chloride (1 : 400, 1 : 200), red copper oxide (1 : 500, 1 : 250), acetic acid (1 : 125), Cheshunt compound (1 : 100), ethyl mercuric bromide (1 : 100,000). Unless otherwise noted proportions are by weight. Dosage was 50 ml. (100 ml. in the case of acetic acid) per crock applied immediately after seeding.

Dust soil treatments—

Semesan (0.1), Semesan Jr. (0.1), black copper oxide (3.0), Du Bay (0.2, 0.3, 0.4, 3 days before seeding), zinc oxide (3.0), copper carbonate (3.0), aluminium sulphate (2.0). Numbers in parentheses denote amount in grams applied per crock.

Combination seed and soil treatments—

The dust soil treatments given above (with the exception of Du Bay and aluminium sulphate) were also applied to crocks sown with seed treated with the corresponding dust. A weekly treatment of 1 : 500 red copper oxide spray, 50 ml. per crock, was used alone and following red copper oxide seed treatment.

The experiment was first carried out using a two-year-old stock of red pine seed which, although germinating fairly well, produced seedlings of low vigour. The result was a very high pre-emergence mortality and practically complete postemergence mortality in the checks and in the more or less non-effective treatments in both soils. The results from the two soils will be discussed separately since analysis of variance gives a highly significant (1%) *F* value for the interaction between soils and treatments.

The disease was particularly severe in the Orono soil, where only five of the 123 crocks contained live seedlings at the end of the experiment. Two were from the Semesan (1 : 200) treatment, one each from mercuric chloride (1 : 400) and (1 : 200), and one from the red copper oxide weekly spray following seed treatment. The latter treatment gave the best control of pre-emergence damping-off, judging from emergence data. The red copper oxide, zinc oxide, and copper carbonate dust seed treatments also reached the 5% level of significance in this respect.

In the Ottawa soil pre-emergence damping-off was much less severe than in Orono soil, seedling emergence being about four times as great. Significant (5% level) reduction of pre-emergence damping-off was achieved by the following treatments: seed treatments involving red copper oxide, Semesan, zinc oxide, and copper carbonate; red copper oxide weekly spray following seed treatment; and combination seed and dust soil treatments involving Semesan, black copper oxide, zinc oxide, and copper carbonate.

Data on postemergence damping-off in Orono soil was not given statistical treatment because of the small numbers of emerged seedlings.

In Ottawa soil, significant (5% level) reduction of postemergence damping-off was afforded by the following soil treatments: Semesan (1 : 200), aluminium sulphate (1 : 40), ethyl mercuric bromide (1 : 100,000), and steam sterilization. Significant reduction was also obtained from the red copper oxide weekly spray following seed treatment.

The 18 liquid soil treatments of the above experiment—i.e., excluding seed treatments, dust soil treatments, and combination treatments—were repeated using a more vigorous stock of red pine seed. Again three replicates of each soil were used, the total number of crocks, including checks and a steam sterilization treatment, being 126.

Unlike the first experiment there was no significant difference between Orono and Ottawa soils with respect to treatment effect on seedling emergence, therefore results on emergence are averaged over the two soils. Two treatments, red copper oxide (1 : 500) and steam sterilization significantly increased emergence, while one, mercuric chloride (1 : 200) caused a significant decrease.

In the case of postemergence damping-off a highly significant (1% level) *F* value was obtained for the interaction, soils \times treatments, hence results will be discussed separately for the two soils. In Orono soils, all treatments, except sulphuric acid (1 : 200), aluminium sulphate (1 : 40), acetic acid (1 : 125), and formalin (1 : 300 and 1 : 50) were effective. The first two are the weaker concentrations of acidifying agents, the higher concentrations of which were effective. In Ottawa soil, all treatments were effective with the exception of aluminium sulphate (1 : 40) and Cheshunt compound (1 : 100).

An interesting result from the repeated experiment is the relatively high resistance of vigorous seedlings (from fresh seed) to pre-emergence damping-off as compared with the weaker seedlings (from old seed) of the original experi-

ment. This is shown particularly by the emergence data from the highly polluted Orono soil. For example, in the original test using weak seed, 9, 20, and 61 seedlings respectively emerged from the three crocks of the treatment involving red copper oxide weekly spray following seed treatment (indicating a fair degree of germinability), while only 1, 0, and 1 seedling respectively emerged from the check crocks (indicating very little resistance to pre-emergence damping-off). In the repeated experiment, emergence from comparable treated crocks was 30, 27, and 20 seedlings, and from check crocks 30, 17, 32. This indicates that actual germination was probably no greater than in the original test, but that the more vigorous seedlings of the repeated test possessed a much greater resistance to pre-emergence damping-off.

Certain results from miscellaneous experiments should be mentioned. In tests of seed treatments using steam-sterilized and non-sterilized soil, treatment effects were completely obscured in the sterilized soil, indicating that damping-off organisms were not appreciably seed-borne. In another experiment, in which soil treatments were withheld until damping-off had commenced, it was shown that treatments that were definitely effective when applied at seeding time were practically ineffective when applied after damping-off had started.

3. Effect of Soil Treatments on pH of the Soil

The results from pH determinations made with a Beckman pH meter on the soil, following treatment, as well as on the treatment solutions themselves, are given in Table I. The effect of the acidifying agents, sulphuric acid and aluminium sulphate, are clearly marked, the former giving pH values averaging just above 3, while the latter gave values mainly in the range 3.5 to 4. Semesan proved to be the most alkaline treatment and gave slightly acid to slightly alkaline soil readings. All other treatments, and also the tap water check, gave slightly acid pH values.

TABLE I

pH DATA FROM DETERMINATIONS MADE IN CROCKS OF SOIL TREATMENTS APPLIED AT SEEDING AND REPEATED AT EMERGENCE (7 TO 12) AND OF SOIL TREATMENTS APPLIED AFTER DAMPING-OFF COMMENCED (13 TO 19)

Treatment chemical (pH in parentheses)	No. of crocks	Range of pH	Average pH
7. Mercuric chloride 6 : 100 (4.89)	12	5.45 - 6.32	5.86
8. Sulphuric acid 1 : 50 (0.47)	12	2.58 - 3.70	3.10
9. Aluminium sulphate 1 : 20 (3.18)	12	2.75 - 4.15	3.78
10. Formalin 1 : 150 (7.30)	12	6.10 - 6.86	6.49
11. Semesan 1 : 400 (11.42)	12	6.44 - 7.81	6.92
12. Tap water check (8.71)	12	6.27 - 6.97	6.60
13. Red copper oxide 1 : 500 (9.22)	4	6.00 - 6.60	6.39
14. Mercuric chloride, as above	4	6.07 - 6.39	6.23
15. Sulphuric acid, as above	4	3.35 - 3.72	3.56
16. Aluminium sulphate as above	4	4.20 - 4.99	4.65
17. Formalin, as above	4	6.10 - 6.43	6.26
18. Semesan, as above	4	6.09 - 7.25	6.49
19. Tap water check, as above	4	6.11 - 6.45	6.23

An attempt to correlate pH of the soil with damping-off control gave an insignificant r value.

COMBINATION SEED AND SOIL TREATMENTS

On the basis of previous results, a factorial experiment was designed in which 27 combinations of seed and soil treatments were tested in both Ottawa and Orono soil. The seed treatments were: red copper oxide, zinc oxide, copper carbonate, and untreated. Each was combined with each of the following soil treatments: untreated, Semesan solution (1 : 200 and 1 : 100), Semesan dust (0.5 gm. per crock), red copper oxide solution (1 : 250), red copper oxide dust (0.5 gm. per crock), and Du Bay dust (0.3 gm. per crock). A steam sterilization treatment, not part of the factorial experiment, was included since it is the common greenhouse procedure. All treatments were replicated twice in each soil, except the untreated and steam sterilized soil which were replicated three times. The experiment involved 126 crocks.

The method of applying treatments was as follows: 25 ml. of solution was applied to appropriate crocks immediately after sowing, repeated one week after sowing, and again (using 50 ml.) four weeks after sowing. Du Bay dust was applied immediately after sowing, other dusts one week after sowing. Dust treatments were not repeated.

The analysis of variance of the results is given in Table II.

TABLE II

ANALYSIS OF VARIANCE OF RESULTS FROM COMBINATION SEED AND SOIL TREATMENTS (GREENHOUSE)

Source of variance	Degrees of freedom	Mean square		
		Emergence	Damping-off	Survival
Between soils	1	3823**	2762**	151
Steam sterilization vs. all other treatments	1	2	5637**	1194*
Between seed treatments	3	453	1175**	261
Between soil treatments	6	3075**	4940**	2268**
Seed treatment \times soil treatment	18	292	423	179
Soil \times steam sterilization vs. all other treatments	1	84	213	317
Soil \times seed treatments	3	408	10	282
Soil \times soil treatments	6	592	377	176
Soil \times seed treatment \times soil treatment	18	327	247	176
Error	68	276	236	186

* Exceeds mean square error, 5% level of significance.

** Exceeds mean square error, 1% level of significance.

The significant difference between soils in relation to emergence and damping-off was due in both cases to consistently higher figures in the results from Orono soil. That this response to Orono soil was not differential with

respect to the various treatments is clearly shown by the complete absence of significant interaction between soils and treatments.

The steam sterilization data for damping-off and survival proved to be significantly better than corresponding data bulked for all other treatments. This significance is almost entirely due to the poor results from Du Bay dust. A fair appraisal of the other soil treatments in comparison with steam sterilization would be that red copper oxide was slightly inferior and Semesan slightly superior.

The significant difference in damping-off results between individual seed treatments resulted from red copper oxide and zinc oxide being distinctly better than copper carbonate in reducing damping-off. In the emergence data (pre-emergence damping-off control) each of the individual seed treatments gave considerably higher averages than the check.

The most significant point in Table II is the highly significant difference between soil treatments for each of the three kinds of data.

In Table III the data of all seed treatments and of both soils have been combined to illustrate the average effects of the various soil treatments on emergence, damping-off, and survival. It will be noted that all three of the Semesan treatments and the liquid red copper oxide treatment were significantly better than the check or the Du Bay treatment, and, except in the case of damping-off, than the red copper oxide dust treatment. Further note should be taken of the fact that Semesan 1 : 100 gave significantly better control of damping-off than any of the other effective treatments.

TABLE III

EFFECT OF SOIL TREATMENTS ON NUMBERS OF EMERGED AND SURVIVING SEEDLINGS AND ON PERCENTAGE POSTEMERGENCE DAMPING-OFF AVERAGED OVER ALL SEED TREATMENTS AND OTTAWA AND ORONO SOILS IN THE GREENHOUSE

Soil treatment	Treatment No.	Emergence	Damping-off	Survival
Check	1	37.8	92.3	2.3
Semesan 1 : 200	2	60.4*	63.1*	24.1*
Semesan 1 : 100	3	58.4*	48.2*	29.9*
Semesan dust	4	66.8*	63.3*	25.3*
Red copper oxide 1 : 250	5	54.9*	77.9*	12.1*
Red copper oxide dust	6	38.6	79.5*	10.0
Du Bay	7	32.1	94.5	1.9
Necessary difference (5% level)	1 and all others	10.7	9.9	8.8
	2 to 7	11.7	10.9	9.6

* Significantly different from the check.

Experimental Results (Nursery)

COMBINATION SEED AND SOIL TREATMENTS AT OTTAWA AND ORONO

Based on the results of the series of greenhouse experiments using Ottawa and Orono soils, a further experiment was designed to test some of the more promising soil and seed treatments, and combinations thereof, under practical conditions at the Ottawa and Orono nurseries in 1941. The seed treatments were red copper oxide, zinc oxide, and untreated, each being combined with each of the following soil treatments: untreated; red copper oxide (1 : 500 weekly and 1 : 250 bi-weekly); Semesan (1 : 300 weekly, 1 : 150 bi-weekly, and dust); aluminium sulphate (1 : 20 one application and 1 : 40 two applications). It will be noted that the differences in concentration for any chemical solution are compensated for by frequencies of application in such a way as to equalize the total amount of the chemical applied in any treatment. Each of the 24 treatments was applied, on a different randomization plan, to a plot in each of three beds at each nursery, making a total of 144 plots. Each plot, $2\frac{1}{2}$ sq. ft. in area, was sown immediately before treatment to 4.0 gm. of 1940 red pine seed. Seeds and chemicals used at both nurseries were from the same stock. The liquid soil treatments were applied at the rate of 250 ml. per sq. ft. at each application, and the dust soil treatment at the rate of 2.5 gm. per sq. ft.

Analyses of variance of the results from both the Ottawa and Orono experiments are given in Table IV. The effects on emergence differed widely between the two nurseries: at Ottawa the seed and soil treatments and interactions between them caused no significant differences, while at Orono highly significant effects resulted. The same is largely true for survival data. The effects of soil treatments on damping-off were significant at both nurseries.

TABLE IV

ANALYSIS OF VARIANCE OF RESULTS FROM COMBINATION SEED AND SOIL TREATMENTS
(OTTAWA AND ORONO NURSERIES)

Source of variance	Degrees of freedom	Mean square					
		Emergence		Damping-off		Survival	
		Ottawa	Orono	Ottawa	Orono	Ottawa	Orono
Between replicates	2	4946	36719*	750	14	26118*	2628
Between seed treatments	2	7672	110875**	296	234	7671	38455**
Between soil treatments	7	28106	36024**	918**	1477*	18277*	32833**
Interaction, seed treatment \times soil treatment	14	15641	23096**	269	182	11502	11628**
Error	46	14612	7279	238	109	7542	3297

* Exceeds mean square error, 5% level of significance.

** Exceeds mean square error, 1% level of significance.

Emergence, damping-off, and survival data (averaged over all seed treatments) in relation to the effect of soil treatment at both nurseries are given in Table V. Both of the Semesan liquid treatments, and red copper oxide (1 : 500 weekly) significantly reduced damping-off, and also significantly increased survival at Ottawa. At Orono, however, only Semesan (1 : 150 bi-weekly) resulted in significant reduction of damping-off. The unfavourable effect of certain soil treatments at Orono appears, from examination of the original data, to be due to a damaging effect resulting from the combining of different chemicals in applying soil treatments following seed treatments.

TABLE V

EFFECT OF SOIL TREATMENTS ON NUMBERS OF EMERGED AND SURVIVING SEEDLINGS AND ON PERCENTAGE POSTEMERGENCE DAMPING-OFF, AVERAGED OVER ALL SEED TREATMENTS, IN OTTAWA AND ORONO NURSERIES

Soil treatment	Emergence		Damping-off, %		Survival	
	Ottawa	Orono	Ottawa	Orono	Ottawa	Orono
Check	478	562	86.4	54.7	74	253
Red copper oxide 1 : 500 weekly	593*	515	71.6*	72.7†	164*	137†
Red copper oxide 1 : 250 bi-weekly	469	575	78.2	71.9†	110	158†
Semesan 1 : 300 weekly	421	403†	60.6*	53.0	172*	192†
Semesan 1 : 150 bi-weekly	470	486	59.0*	39.4*	195*	290
Semesan dust	508	489	76.4	78.9†	126	106†
Aluminium sulphate 1 : 20 one application	517	422†	76.9	64.2	130	165†
Aluminium sulphate 1 : 40 two applications	421	438†	84.9	62.0	70	180†
Necessary difference, 5% level of significance	115	81	14.6	9.9	82	54.0

* Indicates significant favourable effect.

† Indicates significant unfavourable effect.

The interaction between seed and soil treatments as it affects Orono results on emergence and survival is shown in Table VI. Except for one treatment, red copper oxide (1 : 500 weekly), the combination of seed and soil treatments substantially reduced emergence, even though seed or soil treatments alone almost invariably increased emergence. In the case of survival, the combination of seed and soil treatments was without exception highly unfavourable. Here again, the seed and soil treatments when used alone were almost invariably beneficial.

LARGE-SCALE ACIDIFICATION EXPERIMENTS AT ORONO

During the period 1939–1942 inclusive, extensive experiments on the effect of soil acidification on damping-off were carried out at the Orono nursery. Red pine was used in all experiments, the rate of sowing being 1 lb. per seed bed, size 4 × 30 ft. Treatment was applied immediately after sowing. Sulphuric acid was diluted at the rate of 22½ fl. oz. in 30 gal. of water. In 1939 and 1940 each bed was divided, half being treated, the other half left untreated as a check. In 1942 the beds were randomized in lines of five beds. Details of treatments and results are given in Table VII.

These results indicate that the severity of damping-off and the degree of control provided by acidification both vary widely in different years. In 1940, under conditions of very severe damping-off, good control was obtained

TABLE VI

INTERACTION BETWEEN SEED AND SOIL TREATMENTS WITH RESPECT TO EMERGENCE AND SURVIVAL AT THE ORONO NURSERY

Soil treatment	Number emerged (av. of 3 replicates)			Number surviving (av. of 3 replicates)		
	Seed treatment ¹			Seed treatment ²		
	Check	Red copper oxide	Zinc oxide	Check	Red copper oxide	Zinc oxide
Check	507	587	591	188	300	272
Red copper oxide 1 : 500 weekly	502	503	540	156	150	105
Red copper oxide 1 : 250 bi-weekly	657	481	583	186	163	124
Semesan 1 : 300 weekly	478	355	375	263	168	143
Semesan 1 : 150 bi-weekly	541	455	441	373	250	246
Semesan dust	553	473	438	136	86	93
Aluminium sulphate 1 : 20 one applica- tion	524	267	474	219	73	204
Aluminium sulphate 1 : 40 two applica- tions	683	238	363	322	55	163
Mean	556	421	480	231	156	169
Necessary difference	50			33		

¹ Necessary difference, interaction, 140.

² Necessary difference, interaction, 94.

TABLE VII

EFFECT OF ACIDIFICATION TREATMENTS ON DAMPING-OFF OF RED PINE SEEDLINGS AT THE ORONO NURSERY

Time of sowing	Precipita- tion 1 Apr.- 30 June (in.)	Acidifier per bed	Treatment				Check			
			No. of beds	Average germin- ation	Average survival	%	No. of beds	Average germin- ation	Average survival	%
Fall, 1939	9.63	H ₂ SO ₄ 22½ fl. oz.	2½	11532	2874	24.9	2½	13536	680	5.0
Spring, 1940	9.36	H ₂ SO ₄ 22½ fl. oz.	2½	35340	11474	32.5	2½	34015	1793	5.3
Spring, 1941	3.40	H ₂ SO ₄ 22½ fl. oz.	65	13890	3850	27.7	56	14247	4047	28.4
Spring, 1942	12.76	H ₂ SO ₄ 22½ fl. oz.	10	27290	22911	84.0	20	21918	17244	78.7
Spring, 1942	12.76	H ₂ SO ₄ 11½ fl. oz.	10	24590	23010	93.6				
Spring, 1942	12.76	Al ₂ (SO ₄) ₃ 1 lb.	5	18820	15419	81.9				
Spring, 1942	12.76	Al ₂ (SO ₄) ₃ 2 lb.	5	27980	23916	85.5				

in both fall- and spring-sown beds. In 1941, under drought conditions, damping-off was fairly severe, but acidification was completely ineffectual as a control measure. This might be explained on the basis of neutralizing effect of the irrigation water (pH about 8) which was applied by an overhead system. In 1942, under conditions of excessive natural precipitation, damping-off was not very severe, and acidification gave only slight to moderate increases in survival. Lack of better control in this case might be due to the removal of the acidifier by percolation. To go back to the 1940 results, it would seem that the good control provided by acidification might be explained by the assumption that, while the precipitation was sufficient to make irrigation with a more or less alkaline water unnecessary, it was not sufficient to render acidification ineffective through percolation.

General Discussion

The factorial experiments were designed primarily for the purpose of testing combination seed and soil treatments which, it was believed, might be superior to seed or soil treatments applied separately. However, upon combining some of the more effective treatments in the greenhouse and Ottawa nursery, it was found that prior seed treatments had no significant effect on results from superimposed soil treatments, while under nursery conditions at Orono, the combination treatments were significantly inferior to either form of separate treatment.

The most effective treatment proved consistently to be Semesan solution in concentrations of 1 : 100 to 1 : 150 applied to the soil at time of sowing and at emergence in the greenhouse, or at bi-weekly periods from sowing until four or five weeks after emergence in the nursery. Under greenhouse conditions, this treatment gave results equal to or slightly better than steam sterilization of the soil.

Next in efficiency was the soil treatment with red copper oxide suspension in concentrations of 1 : 250 to 1 : 500 applied according to the schedule given in the preceding paragraph. The use of this chemical as a seed and soil disinfectant has been intensively studied by Horsfall and co-workers (3).

A number of fungicidal dusts proved efficient as seed treatments, especially red copper oxide and zinc oxide used under greenhouse conditions. As a group, dust treatments proved greatly superior to liquid treatments as seed disinfectants.

The general results of the experiments also show that the fungicidal agents provided more consistent damping-off control than did acidifying agents. This is not in accord with the consensus of recent papers (2, 4, 5) in recommending acidification of the soil.

The fact that acidification gave little to no control in seasons of excessive rainfall or drought is presumed to be related to reduction of acidity to a non-effective level, by percolation in the one case and through application of slightly alkaline irrigation water in the other. Acidification apparently does not kill

the damping-off organisms, but prevents development by conditioning an unfavourable environment. Therefore, upon removal or neutralization of the acid, damping-off quickly develops. Fungicides on the other hand presumably kill the organisms and, therefore, provide a more positive control, less influenced by environmental conditions.

The disclosure in the present work of a strong indication that seedlings from two-year-old red pine seed of somewhat reduced vitality were far more susceptible to damping-off than those from fresh seed is considered to be a point of some importance. Since it is frequently necessary to store coniferous seed for two or three years owing to the spread of good seed years, this point brings up a storage problem that should not be disregarded.

Acknowledgments

The authors gratefully acknowledge the valuable assistance rendered by Dr. J. W. Hopkins and Staff in the statistical analysis of the data; by Messrs. E. C. Bradley, H. W. Holtz, L. Brown, and E. Emond in the experimental phases of the work; and by Dr. J. G. Malloch in preparing the manuscript.

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CONTRIBUTIONS TO A STUDY OF THE FUNGOUS FLORA OF NOVA SCOTIA

VI. PYRENOAMYCETES¹

BY LEWIS E. WEHMEYER²

Abstract

The 170 species and two varieties of fungi listed from the province are distributed in 70 genera as follows: Perisporiales—seven species and one variety in five genera; Hypocreales—29 species in 12 genera; Sphaeriales—129 species and one variety in 49 genera; Dothideales—five species in four genera. The following species are described as new: *Leptosphaeria anisomeres*, *Physalospora Laricis*, *Chaetosphaeria multiseptata*, *Massaria saliciformis*, *Cryptospora aurantiaca*, *Diaporthe quadruplex*, *Xylaria coprophila*. The following new combinations are made: *Bombardia* (*Sordaria*) *lutea* (E. & E.), *Pseudotrichia* (*Sphaeria*) *viridicoma* (Cke. and Pk.), *Pleospora* (*Teichospora*) *nitida* (E. & E.), *Apiognomonina* (*Gnomoniella*) *guttulata* (Starb.), *Gibberidea* (*Cucurbitaria*) *alnea* (Pk.).

The present paper is a report upon the pyrenomycetes collected over a series of summer field trips to the province of Nova Scotia. The reader is referred to the first paper of this series (20) for a description of the province in general and the localities mentioned, in particular.

These collections, again, represent only a small fraction of the species actually occurring in this region, for they were merely picked up in the course of general collecting. As very few pyrenomycetes have been reported from the province, however, they represent mostly new records.

The older arrangement of orders and families, as given by Winter (24), is followed. It is recognized that probably the bulk of the species placed in the Sphaeriales have a "perithecial" development considered to be pseudo-sphaeriaceous or dothideaceous. These terms are still largely theoretical, however, and apply to only a few species which have been studied in detail. There has never been any approach to an arrangement of the great bulk of existing species in the light of these theories and these terms are meaningless as taxonomic units for any thoroughgoing arrangement of species. The Allantosphariaceae and Diaporthaceae of Höhnelt are recognized because they represent a distinct advance in the arrangement of the stromatic Sphaeriales and because the genera included have been assigned to these families. The few species of *Anthostoma*, *Fenestella*, *Valsaria*, etc. have been arbitrarily placed in the Diaporthaceae for want of a better repository.

Seaver's (16) arrangement of the Hypocreales has been followed, inasmuch as it is the only complete American account of this order.

¹ Manuscript received June 22, 1942.

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Perisporiales

Capnodiaceae

Phaeocryptopus nudus (Pk.) Petr. (*Adelopus balsamicola* (Pk.) Theiss.). On killed needles of living trees of *Abies balsamea* (L.) Mill., Upper Brookside, June 27, 1931 (300a) and June 19, 1933 (1673).

Petrak (15) gives a full discussion of this genus and separates the fungus fruiting on the living needles of *Pseudotsuga* from this one on the killed needles of *Abies*.

Scorias spongiosa (Schw.) Fr. On living *Alnus* sp., Salmon River, July 14, 1931 (1018). Both the pycnidial and perithecial stages were found on this host.

Erysiphaceae

Erysiphe Galeopsidis DC. On *Chelone* sp., Upper Brookside, Aug. 28, 1931 (1420); Victoria Park, Sept. 5, 1935 (1682).

Erysiphe graminis DC. On *Agropyron repens* (L.) Beauv., Upper Brookside, June 29, 1931 (458).

Erysiphe Polygoni DC. On *Ranunculus acris* L., Upper Brookside, Aug. 28, 1931 (1423) and Aug. 31, 1931; on *Trifolium* sp., Onslow, Colchester Co., Aug. 31, 1931 (1497).

Microspheera Alni (Wallr.) Salm. On *Alnus incana* (L.) Moench, Salmon River, Sept. 7, 1931 (1478); on *Syringa vulgaris* L., Upper Brookside, Sept. 11, 1931 (1494).

var. **Vaccinii** (Schw.) Salm. On *Epigaea repens* L., Grande Anse, Richmond Co., Aug. 3, 1931 (1238).

Phyllactinia corylea (Pers.) Karst. On *Alnus incana* (L.) Moench, Salmon River, Sept. 7, 1931 (1478).

Hypocreales

Nectriaceae

Letendraea luteola E. and E. On decayed wood, Salmon River, July 14, 1931 (1026).

Nectria coccinea (Pers.) Fr. On living bark and on cordwood of *Fagus grandifolia* Ehrh., Truro, July 11, 1929 (37); Salmon River, July 15, 1931 (1041).

This is the species of *Nectria* associated with the beech bark disease in the northeast. Ehrlich states that it differs from Seaver's (16, p. 21) description of *Creonectria coccinea* in the smaller spores, but is probably a form of *Nectria coccinea*.

Nectria (Creonectria) Coryli Fck. On *Salix* sp., Upper Brookside, July 13, 1931 (1003).

Nectria (Creonectria) spp.

The stroma-forming species of *Nectria* of North America are so poorly known and their nomenclature so confused, that it has been impossible to place several collections. The description of these collections is given here in the hope that they may be properly placed at a later date.

A—On *Betula* spp., Economy Lake, June 16, 1926 (P73a); Victoria Park, Truro, Aug. 15, 1933 (1631).

Perithecia bright, then dull red, 300–350 μ in diameter, crowded upon an orange-red, erumpent stroma, spheric at first, smooth, becoming collapsed. Ascospores variable in shape and size, cylindric to ellipsoid, straight or slightly curved, usually constricted, two-celled at first, in age becoming three- to four-celled, (13) 15–22 \times 4–7 μ . In both these collections the ascospores were accompanied by numerous cylindric to allantoid, hyaline, one-celled conidia, 5–7 \times 1.5–2 μ . These may be sprout conidia from the ascospores, but no actual attachment was seen. In No. 1631, long, lunate, *Fusarium*-like conidia were seen. These were 40–63 \times 2.5 μ .

B—On *Acer* sp., Salmon River, Oct. 2, 1926 (96), leg. A. R. Prince.

Perithecia pale red to orange-red, 300 μ in diameter, thickly clustered on a cream to yellowish stroma, very slightly roughened, with an umbilicate ostiole, finally somewhat collapsed. Asci 53–63 \times 8.5–10 μ . Spores mostly long-cylindric, straight or slightly curved, very slightly or not at all constricted, two-celled, septum faint, hyaline, 12.5–14.5 \times 3.5–4.5 μ .

C—On *Betula papyrifera* Marsh., Upper Brookside, July 13, 1931 (1010).

Perithecia bright red becoming deep blood red, 200–250 μ in diameter, with a prominent ring-like thickening about the upper portion, through which the ostiole is erumpent. Asci cylindric 50–60 \times 3.5 μ . Spores uniseriate, ellipsoid, hyaline, two-celled with a very faint septum, ends rounded, 7–9 \times 2.5–3.5 μ .

These collections were submitted to Dr. Seaver for an opinion. He suggests that A and B may be *Creonectria purpurea* (L.) Seav. The spores are of a size found in this species, but the perithecia do not show the coarse roughness characteristic of it. C he places as *Nectria pilhoides* E. and E., which is also the writer's opinion. Wollenweber (25, p. 185) gives this species as a synonym of *N. appianata* Fck.

Nectria episphaeria (Tode) Fr. On sphaeriaceous stromata on *Betula*, Wolfville, June 26, 1926 (P25a); Upper Brookside, July 31, 1931 (386a); on *Corylus cornuta* Marsh., Earlton Rd., Aug. 22, 1931 (308b); on *Dermatea* on *Abies*, Pictou Rd., June 30, 1931 (530).

Common on stromata of various fungi particularly the Sphaeriales.

Nectria lactea Ell. and Morgan. On myxomycete sporangia and on wood upon which they are growing, Upper Brookside, July 17, 1931 (1071 and 1071a), leg. A. H. Smith.

The perithecia are minute, white, and covered with a cottony tomentum, giving them the appearance, superficially, of a parasitic hyphomycete.

Nectria Peziza (Tode) Fr. On *Polystictus* sp., Upper Brookside, July 24, 1929 (100); on decayed wood, Upper Brookside, July 16, 1931 (1064 and 1065).

Nectria sanguinea (Bolt.) Fr. On conifer stump, Earlton Rd., Aug. 26, 1931 (1411).

This collection is placed here on account of its occurrence on decorticated wood. It is scarcely distinguishable from *N. episphaeria*. Seaver distinguishes *N. sanguinea* from *N. episphaeria* by the broad-fusoid (10–12 \times 4–5 μ) instead of narrow-fusoid (9–12 \times 4–6 μ) spores, but there is obviously very little difference.

Passerinula candida Sacc. Parasitic on stromata of some discomycete on *Abies balsamea*, New Glasgow Rd., July 25, 1931 (1148).

Perithecia immersed in the disk-shaped, black, leathery, partially erumpent stromata of a *Dermatea* or *Tympanis*, 200–250 μ in diameter, walls reddish, parenchymatous, appearing like those of a *Nectria*, erumpent as small, papillate, reddish-black ostioles. Asci cylindric, 90–130 \times 10–11 μ . Spores uniseriate to irregularly biseriate, ellipsoid to fusoid-ellipsoid, ends blunt, two-celled, granular, hyaline at first, becoming brownish, (14) 17–21 \times 8–9 μ .

Passerinula candida, which is reported as parasitic on pyrenomycete stromata, has not been seen. The description of that species differs only in the different ostioles, smaller perithecia ($\frac{1}{4}$ – $\frac{1}{2}$ mm.), the attenuate-stipitate asci and the four-guttulate spores. Whether or not these are varietal or specific differences can be determined only by comparison with authentic material of this species.

Scoleconectria balsamea (Cke. and Pk.) Seav. On *Abies balsamea*, New Glasgow Rd., July 25, 1931 (1147 and 1148).

The spores of these collections measure 14–28 \times 3.5–5 μ .

Scoleconectria scolecospora (Bref.) Seav. On *Abies balsamea*, Moore's Lake, Halifax Co., July 6, 1929 (29); Earlton Rd., Aug. 22, 1931 (362a).

The spores of this species are 30–60 \times 2–3 μ . Both this and the preceding species show numerous sprout conidia in the asci, and are fairly common on fir.

Hypocreaceae

Byssonectria violacea (J. C. Schm.) Seav. On *Fuligo* sp., Aug. 15, 1931 (1323).

Chromocrea gelatinosa (Tode) Seav. On *Alnus* sp., Aug. 14, 1935 (1680).

The stromata are flat-pulvinate, cream-yellow at first, becoming greenish because of the exuded spores which are dark green to green-brown at maturity.

Claviceps purpurea (Fr.) Tul. On *Agropyron repens*, Onslow, Colchester Co., Aug. 31, 1931 (1440), sclerotia only.

Cordyceps capitata Link. On *Elaphomyces* sp., Wentworth Valley, Cumberland Co., Aug. 29, 1931 (1433), leg. A. H. Smith.

- Cordyceps militaris** (L.) Link. On pupa, Lake O'Law, Inverness Co., Aug. 6, 1931 (1070a); on cocoons, Upper Brookside, Aug. 16, 1927, leg. A. R. Prince (6217); July 17, 1931 (1070), leg. A. H. Smith.
- Cordyceps ophioglossoides** (Ehr.) Sacc. On *Elaphomyces* sp., Northeast Margaree, Sept. 4, 1927 (388).
- Cordyceps stylophora** Berk. and Br. On beetle larvae, Upper Brookside, July 25, 1931 (1167), det. E. B. Mains.
- Cordyceps viperina** Mains. On beetle larvae, Upper Brookside, July 25, 1931 (1213); Earltown Rd., Aug. 26, 1931 (1213a).
These are two of the collections upon the basis of which Mains (11) described this species.
- Hypocrea citrina** (Pers.) Fr. On *Fomes pinicola* (Swartz) Cke., Upper Brookside, July 4, 1931 (386); on *Fomes applanatus* (Pers.) Gill, Princeport, Colchester Co., July 9, 1931 (468).
- Hypocrea patella** Cke. and Pk. On decayed beech log, Folley Lake, July 20, 1931 (1094).
The stromata vary from almost white to bright yellow or ochraceous.
- Hypocrea rufa** (Pers.) Fr. On *Fagus grandifolia*, Upper Brookside, July 11, 1931 (494); on *Alnus* sp., Salmon River, July 15, 1931 (494a).
Quite common on the moist surface of down logs.
- Hypomyces apiculatus** (Pk.) Seav. On humus of old stump, Salmon River, Sept. 7, 1931 (1475).
- Hypomyces aurantius** (Pers.) Tul. On *Irpex* sp. on birch, Upper Brookside, July 24, 1929 (99); on *Boletus*, Killag Mines, July 31, 1931 (1178); on *Polystictus versicolor* (L.) Sacc., Upper Brookside, Aug. 24, 1931 (1397).
- Hypomyces chrysospermus** (Bull.) Tul. On an agaric, Salmon River, Aug. 18, 1931 (1350); on *Boletus*, Folley Lake, Aug. 29, 1931 (1455), leg. A. H. Smith.
Sydow (18, p. 186) described the genus *Apiocrea* for those species of *Hypomyces* whose spores have two cells of unequal size and cited the species *Hypomyces chrysospermus* (Pers.) Tul., *H. hyalinus* (Schw.) Tul., and *H. Tulasneanus* Plowr. as belonging in this genus. Seaver (16) gives *H. chrysospermus* as having spores slightly roughened at maturity and $12-15 \times 4 \mu$. European descriptions of this latter species give its spores as $21-30 \times 5-6 \mu$. The spores of *H. Tulasneanus* are given as $20-25 \times 8 \mu$. These two collections from Nova Scotia have the same appearance, but the ascospores of No. 1350 are large, $17.5-21 \times 4-5 \mu$, have a long appendage at each end, and are smooth and somewhat constricted at the septum. The conidia of the *Sepedonium* stage are comparatively short-tuberculate. This appears to be the European *H. chrysospermus*. No. 1455 has shorter spores, $9-12 \times 2.5-3.5 \mu$, with shorter appendages, and the conidia have longer, cylindric tubercles. This may be the *H. chrysospermus* of Seaver.
- Hypomyces lactifluorum** Schw. On an agaric, Upper Brookside, Aug. 31, 1925, leg. A. R. Prince (1099); quite common.
- Hypomyces polyporinus** Pk. On *Polystictus versicolor*, Earltown Rd., Aug. 19, 1931 (1368).
- Hypomyces rosellus** (Alb. and Schw.) Tul. On decayed *Poria*, Upper Brookside, Aug. 24, 1931 (1396).
- Peckia viridis** (Alb. and Schw.) Sacc. On *Russula* spp., Upper Brookside, July 16, 1931 (1058); Sept. 4, 1931, leg. A. H. Smith.
- Podostroma alutaceum** (Pers.) Atk. On moss and duff under conifers, Salmon River, Aug. 13, 1931 (1293), leg. A. H. Smith; Aug. 18, 1931 (1293a).

Sphaeriales

Chaetomiaceae

- Chaetomium globosum** Kze. In gross culture of dung (grouse?), Upper Brookside, 1931.
Although the tips of the hairs were more spirally coiled than described by Chivers (4, Pl. 15, Fig. 11) for *C. globosum*, two types of hairs as in *C. cochlioides* could not be distinguished.
- Chaetomium indicum** Corda. In gross culture of deer dung, Upper Brookside, (C2) det. by Ralph Bennett.

Sordariaceae

Bombardia coprophila (Fr.) Kirschst. On cow dung, Northeast Margaree, Sept. 4, 1927 (P434); Portapique Beach, July 26, 1933 (1595); on porcupine dung, Mt. Thom, Aug. 15, 1931 (1313).

Bombardia lutea (E. and E.) comb. nov. (*Sordaria lutea* E. and E., J. Mycol. 3:118. 1887). On decayed wood, Killag Mines, July 30, 1931 (1187); Upper Brookside, July 31, 1931 (1187a).

As has been pointed out by Cain (2, p. 66), this species has the asci of the genus *Bombardia*. They are long-cylindric, tapered toward the apex and have a cylindric plug in the tip. It is transferred to *Bombardia* for this reason. Ellis (5, p. 132) gives the asci as 12- to 16-spored and transfers the species to the genus *Philocopra* on this basis. These collections appear to have eight-spored asci. Ellis also fails to mention or figure the secondary appendages, which were seen on these spores, and which appear at each end, and are filiform, 15-20 μ long. The perithecia are not stromatic in this fungus, unless the outer layer of yellowish tomentum of the thick-walled perithecia be considered as a stroma.

Sordaria appendiculata Auersw. On rabbit dung, Green Oaks, Colchester Co., July 12, 1929 (178); New Glasgow Rd., July 25, 1931 (1145); Upper Brookside, July 27, 1931 (1145a); on porcupine dung, Upper Brookside, Aug., 1931 (1492).

Sordaria fimicola (Rob.) Ces. and de Not. On moose dung, Killag Mines, July 30, 1931, det. Ralph Bennett.

Sporormia ambigua Niessl. On horse dung, Earltown Rd., Aug. 22, 1931 (1393).

Trichosphaeriaceae

Aplosporina Collinsii (Schw.) Höhn. Causing a witches' broom on *Amelanchier* sp., Bass River, July 26, 1935 (1752).

Chaetosphaeria multiseptata sp. nov. (Figs. 1, 2).

Perithecia superficial, gregarious to crowded, spheric, 250-300 μ in diameter, black, carbonous, brittle, sparsely covered with short, stiff, dark brown, septate hairs, 30-70 \times 5-6 μ . Subiculum lacking or very sparse. Ostiole short, conic-papillate. Asci clavate with a refractive ring in the slightly thickened apical wall, 90-125 \times 10.5-12.5 μ . Paraphyses filiform, hyaline, persistent. Spores biseriate, oblong-fusoid to cylindric, straight or somewhat curved, brown, six- to eight-celled with the end cells small, cap-like and hyaline, not constricted at the septa, 32-39 \times 5-6 μ , with a large guttule in each cell.

Type: Herbarium L. E. Wehmeyer, on decayed stump, Upper Brookside, Colchester Co., N.S., 26, VII, 1929, leg. L. E. Wehmeyer (No. 78).

Although the perithecia are very sparsely setose and without a subiculum, the spores of this species are typical of *Chaetosphaeria*. The only closely related species are *C. Rehmsiana* (Henn.) Kirschst. and *C. caelestina* Höhn, both of which are larger throughout, are described as having six-celled spores, and occur on tropical hosts.

Chaetosphaeria multiseptata sp. nov. Perithecia superficialia, gregaria vel dense aggregata, sphaerica, 250-300 μ diametro, nigra, carbonea, sparse pilis rigidis atrobrunneis septatis 30-70 μ longis, 5-6 μ diametro vestita. Subiculum nullum vel sparsum. Ostiolum breve, conice papilliforme. Asci clavati, 90-125 μ longi, 10.5-12.5 μ crassi, apice annulo refractivo praediti et membrana paulum incrassata. Paraphyses filiformes, hyalinae, persistentes. Sporae biseriatiae oblonge fusiformes vel cylindricae, rectae vel leviter curvatae, brunneae, 6- vel 8-cellulae, ad septa non constrictae, 32-39 μ longae, 5-6 μ crassae, cellulis uniguttulatis, terminalibus hyalinis, parvis, hemisphaericis.

Specimen typicum legit L. E. Wehmeyer, No. 78, in ligno putrido prope "Upper Brookside, Colchester Co." in Nova Scotia, 26, VII, 1929.

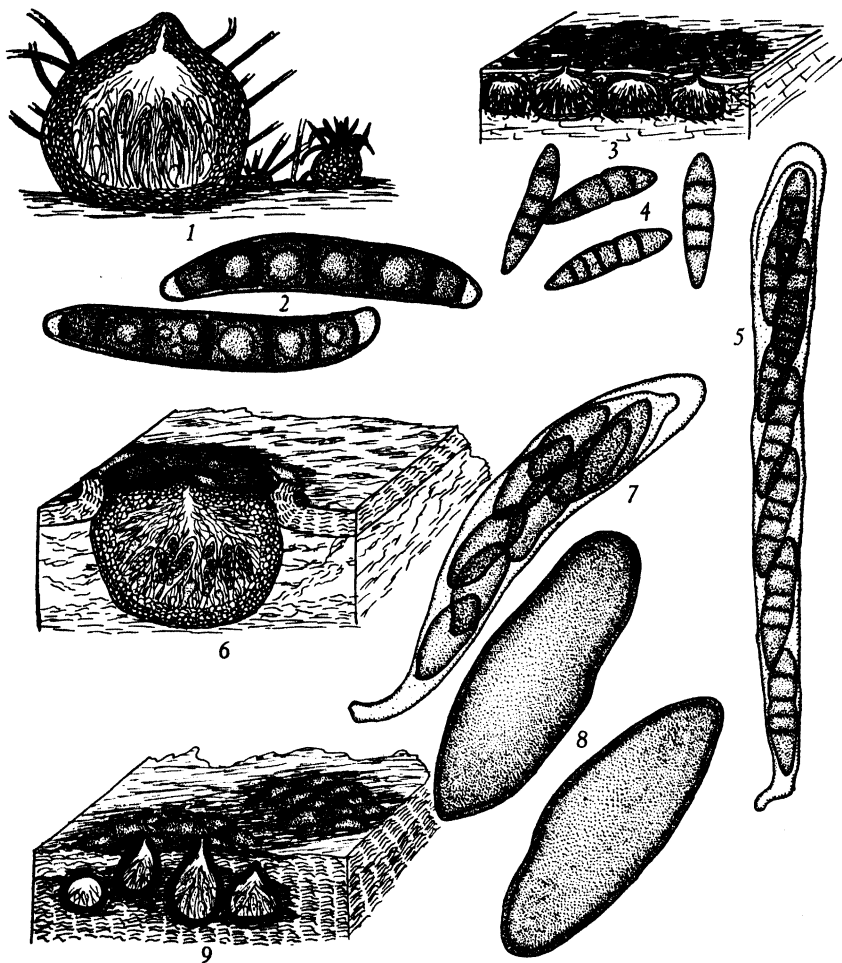
Species subiculo nullo etiamque sparsitate setorum aberrans sed sporis *Chaetosphaeriae* typicis, affinis est *C. Rehmsianae* et *C. caelestinae*, speciebus tropicalibus omnino grandioribus, cum sporis 6-cellulis.

Helminthosphaeria Clavariae (Tul.) Fck. Parasitic on *Clavaria cristata* Holmsk. ex Fr., Upper Brookside, July 27 and Aug. 27, 1931 (1164 and 1669); on *Clavaria cinerea* Bull., Upper Brookside, Aug. 20, 1931 (1374) leg. A. H. Smith.

The dark brown hyphae of the parasite can be seen penetrating throughout the tissue of the host, and cause a deformation and blackening of the stalks. At the surface these parasitic hyphae protrude and bear the conidial stage, *Scolecotrichum Clavarium* (Desm.) Sacc.

Herpotrichia pezizula (B. and C.) E. and E. On *Acer spicatum* Gam., Upper Brookside, Aug. 12, 1931 (1288).

This is accompanied by the conidial stage, *Helicoma Curtisii* Berk.



FIGS. 1 AND 2. *Chaetosphaeria multiseptata* Wehm.

FIG. 1. Vertical section of perithecium and perithecial primordia. FIG. 2. Ascospores.

FIGS. 3 TO 5. *Leptosphaeria anisomeres* Wehm.

FIG. 3. Radial and surface views of perithecia. FIG. 4. Ascospores showing irregular septation. FIG. 5. Ascus.

FIGS. 6 TO 8. *Physalospora Laricis* Wehm.

FIG. 6. Radial section and surface view of perithecium on *Larix*. FIG. 7. Ascus. FIG. 8. Ascospores.

FIG. 9. Radial section and surface view of perithecial clusters of *Massaria saliciformis* Wehm.

Lasiophaeria hirsuta (Fr.) Ces. and de Not. On decayed log, Mt. Thom, Aug. 10, 1931 (1253).

The spores of this material are one-celled, hyaline at first, becoming pale brown and four-guttulate, $55-59 \times 4-5 \mu$. The one-celled condition of the spores suggests *L. strigosa* (Alb. and Schw.) Sacc. but the spores are too long for that species. The septa

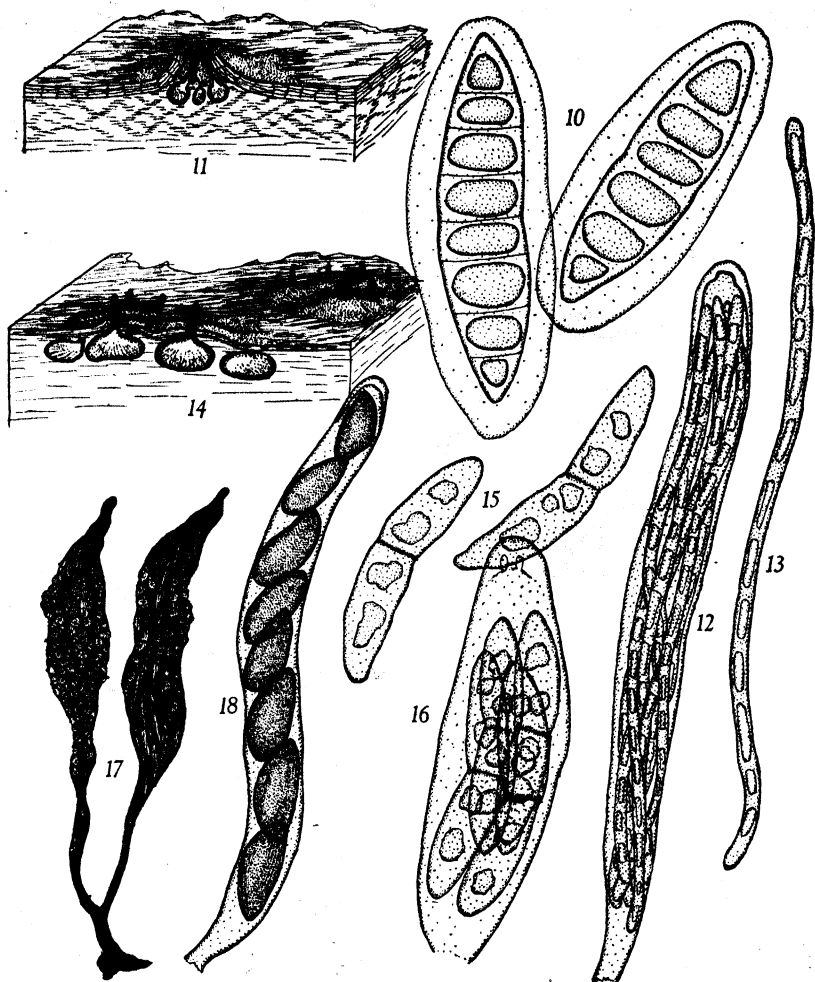


FIG. 10. Ascospores of *Massaria saliciformis* Wehm.

FIGS. 11 TO 13. *Cryptospora aurantiaca* Wehm.

FIG. 11. Radial section of perithecial stroma. FIG. 12. Ascus. FIG. 13. Ascospore.

FIGS. 14 TO 16. *Diaporthe quadruplex* Wehm.

FIG. 14. Radial section and surface views of perithecial stromata. FIG. 15. Ascospores. FIG. 16. Ascus.

FIGS. 17 AND 18. *Xylaria coprophila* Wehm.

FIG. 17. Habit of perithecial stromata. FIG. 18. Ascus with ascospores.

in the spores of *L. hirsuta* are usually faint or absent in most collections, and this is probably that species. The asci are of the *Bombardia* type.

Pseudotrachia viridicoma (Cke. and Pk.) comb. nov. (*Sphaeria viridicoma* Cke. and Pk. Rept. New York State Museum, 29 : 64. 1878.) On wood infected by some pyrenomycete, Upper Brookside, Aug. 12, 1931 (1174).

The nomenclature of this fungus was treated by the writer (22, p. 57) previously, under the name of *Pseudotrachia aurata* (Rehm.) Wehm. It has since been noted that Kauffman (9, p. 187) discussed the same fungus from northern Michigan and placed it as *Lophiostoma viridicoma* (Cke. and Pk.) Kauff. Kauffman, as did Petrak, emphasized the slit-like character of the ostiole and placed the fungus in the Lophiostomataceae. The ostioles are definitely conic, however, and the fact that the apical opening is sometimes slit-like instead of pore-like is not sufficient reason for placing it in this family. The spores are not of the type found in the true species of *Lasiosphaeria*, as Kauffman recognized. It seems better to retain the fungus in Kirschstein's new genus *Pseudotrachia* which was created for it. The species name *aurata* is preceded by *viridicoma*, however, and a revised synonymy of the species is presented.

Pseudotrachia viridicoma (Cke. and Pk.) comb. nov.

Sphaeria (Villosae) viridicoma Cke. and Pk., Rept. New York State Museum, 29 : 64. 1878. (also 26 : 87. 1874.)

Lasiosphaeria viridicoma Sacc., Sylloge Fungorum, 2 : 193. 1883.

Thyridaria aurata Rehm., Ann. Mycol. 10 : 392. 1912. (also 12 : 172.)

Lophiostoma viridicoma Kauff., Mich. Acad. Sci. (Papers) 9 : 189. 1929.

Pseudotrachia stromatophila Kirschst., Ann. Mycol. 37 : 125. 1939.

Pseudotrachia aurata Wehm., Mycologia, 33 : 60. 1941.

Kauffman's collection is on decorticated wood which had obviously been infected by some other pyrenomycete, as shown by the blackened zones within, and on the surface of the wood. Reliquae Farlowianae No. 42, of *Lasiosphaeria viridicoma* is also on stromatic disks of one of the Sphaeriales. These collections confirm the opinion that this fungus is of wide occurrence on wood and bark infected by pyrenomycetes and has probably been described under other names.

Melanommeeae

Bertia moriformis (Tode) de Not. On decorticated stick, Upper Brookside, July 11, 1931 (495); on *Acer spicatum* Lam., New Glasgow Rd., July 25, 1931 (1152).

Melanomma disjectum (Karst.) Sacc. On decayed log (fir?), Upper Brookside, July 19, 1929 (168).

This collection fits the description of this species almost exactly. The fungus is of the *Leptosphaeria* type, however. The perithecia are immersed within the wood fibres when young and later are erumpent. The spores are fusoid-ellipsoid, pale yellow, straight or slightly curved, 20-22 \times 4-4.5 μ and have the second cell from the apex slightly enlarged, as is so often found in *Leptosphaeria*. The paraphyses are septate, cellular strips of interthelial tissue.

Melanomma Pulvis-pyris (Pers.) Fck. On decorticated wood, Upper Brookside, Aug. 11, 1931 (1275); Victoria Park, July 18, 1933 (1591).

The species of *Melanomma* are difficult to separate. Most collections are lumped under the ubiquitous and variable *M. Pulvis-pyris* on the one hand, and numerous species have been described with doubtfully valid differences on the other. Winter (24, p. 249), Ellis (5, p. 181), and most authors give the spores of this species as 16-18 \times 4-6 μ . Chesters (3, p. 119), after examining a large series of collections, found the spores to vary from 11.5-18 \times 4-6 μ , with most spores being 14-16 \times 5 μ , and very few below 14 μ in length. No. 1591 has spores 14-16 \times 4-4.5 μ , which are typical of this species. In No. 1275 the spores are somewhat smaller (12.5-16 \times 3-3.5 μ) and the second cell is usually somewhat swollen. The perithecia are also more hemispheric, and somewhat sunken in the slightly blackened surface of the substratum. It approaches the *Leptosphaeria* type of organization. It was thought that this might be *M. asterostomum* E. and E., on beech roots, but material of this species, kindly sent by Dr. Dearnest, shows that species to have more superficial, somewhat elongate perithecia and spores 12.5-14 \times 3.5-5 μ , without a swollen cell. No. 1275 might be placed in *M. Aspergrenii* Fck.

Melanomma subsparsum Fck. On dead stick, Victoria Park, July 6, 1931 (409); on bark of *Betula* sp., Upper Brookside, July 10, 1931 (476); Green Oaks, Colchester Co., July 9, 1931 (472); on bark of *Sorbus americana* Marsh., Victoria Co., Aug. 5, 1931 (1244).

Under this name are grouped a number of collections of a sort which are commonly found cited as *M. Pulvis-pyrus*, but in which the spores are definitely smaller than given for this latter species. The spore measurements of these collections are: No. 409, $12.5-13 \times 3.5-4.5 \mu$; No. 476, $12-14.5 \times 4-5 \mu$; No. 1244, $10.5-12.5 \times 3.5 \mu$. These fall within the range given for *M. fuscicolum* by Chesters (3, p. 123), but these collections do not have the prominent ostiole nor the biseriata spores of that species.

Rosellinia conglobata (Fck.) Sacc. var. *microtricha* (Feltg.) Höhn. On *Fagus grandifolia*, Middle River, Victoria Co., Aug. 5, 1931 (1229).

The spores of this collection are $8-10.5 \times 4.3-5.5 \mu$. They are somewhat smaller than those of *R. ligniaria* (Grev.) Fck. and somewhat larger than those of *R. velutina* Fck., although the perithecia have the short stout spines of these species. This material differs from *R. pulveracea* in the spiny perithecia and smaller spores, but Thuemen's Mycotheca Universalis No. 861 of *R. pulveracea* forma *Sambuci-racemosae* shows these short spines, but has the spores of *R. pulveracea* ($10.5-12 \times 5.5-7.5 \mu$). Saccardo* gave *R. Sordaria* (Fr.) Rehm. as having spiny perithecia and the spores of *R. velutina* ($7-8 \times 5 \mu$), but Winter (23, p. 100) states that *R. Sordaria* has smooth perithecia. Feltgen (6, III, p. 288) described a variety, *microtricha* of *R. Sordaria* with short spines, and spores $7-10 \times 3-5.5 \mu$, on *Fagus*. Höhnelt (7, p. 1198) states that this variety is on *Corylus*, has nothing to do with *R. Sordaria*, and is a variety of *R. conglobata*, with similar spores ($9-12 \times 5-7 \mu$). An examination of the type material of this variety, kindly sent by Doctor Linder, proves it to be identical with the Nova Scotian material. Its spores are $7.8-9.5 \times 4.5-5.5 \mu$, and the perithecia have similar short spines.

Zignoella aterrima (Fck.) Sacc. On decorticated hardwood, Middle River, Victoria Co., Aug. 5, 1931 (1227).

Perithecia $200-300 \mu$ in diameter, with base somewhat immersed. Spores fusoid, hyaline, one-celled at first, becoming two-celled and four-guttulate, $12.5-16.5 \times 2.5-3.5 \mu$, accompanied by many small ellipsoid, hyaline, one-celled spores, which might be sprout conidia, although no actual budding of the ascospores was seen. The perithecia are accompanied by stiff, upright, dark brown, sparsely septate conidiophores, 5μ in diameter. These bear, at their tips, clusters of curved, cylindric conidia which are at first one-celled, hyaline, then two-celled, pale brown, and finally four-celled and brown. They are $12.5-18 \times 4.5-5.5 \mu$. This fits the description of *Zignoella aterrima* very well, except that the spores ($12 \times 4 \mu$) and conidia ($8-10 \times 6 \mu$) are somewhat smaller in that species.

Zignoella Pulviscula (Curr.) Sacc. On wood of *Fagus grandifolia*, Upper Brookside, July 11, 1931 (489); Aug. 11, 1931 (1275).

The spores in these collections are long cylindric-fusoid, usually curved, hyaline, one-celled, many-guttulate, and $25-37 \times 3.5 \mu$. Although not yet mature enough to show septa, their measurements are greater than those given for this species. The wood surface bears stiff, upright, dark brown, septate conidiophores which, in No. 489, bear four to many-celled conidia, which are ellipsoid to cylindric, sometimes curved, and have the central cells coloured brown, whereas the end cells remain hyaline. They measure $21-45 \times 7 \mu$.

Lophiostomeae

Lophidium compressum var. *microscopicum* Karst. On *Betula* sp., Salmon River, July 15, 1931 (1038).

The spores of this collection measure $14-18 (20) \times 5.5-6 \mu$ and are rather small for the measurements of this species as given by Winter, but fit those given for the variety. The perithecia are also superficial as given for the variety, and, when young, show a sparse covering of fine short hairs which soon disappear however.

Amphisphaeriaceae

Amphisphaeria Juniperi Tracy and Earle. On weathered wood of fir, Portapique Beach, July 31, 1933 (1603); on rotten wood (conifer?), Victoria Park, June 21, 1933 (1674).

Of a number of Amphisphaerias described on coniferous wood, this collection fits best the above species. The perithecia are conic with a flattened base, $450-500 \mu$ in diameter and have thick parenchymatous walls. The asci are clavate with a thickened apical wall, $105-135 \times 25-30 \mu$. The spores are biseriata, ellipsoid-fusoid, two-celled, brown, constricted at the septum, with the lower cell often smaller and tapered, $32-39 \times 11-12.5 \mu$.

* *Sylloge Fungorum*, 1: 270. 1882.

No. 1674 is accompanied by a conidial stage consisting of upright, superficial, cylindric-conic, black pycnidia with a large ring-like ostiole and a central mass of hyaline conidia budded off from the peripheral walls. The conidia are fusoid-ellipsoid, one-celled, and measure $4.3\text{--}5.3 \times 1.5\text{--}2 \mu$.

Strickeria obducens (Fr.) Wint. On *Spiraea* sp., Salmon River, Truro, Sept. 3, 1929 (268).

The spores of this collection ($23\text{--}25 \times 7\text{--}9 \mu$) are slightly smaller than those given for *S. obducens*, but this is probably that species.

Strickeria villis (Fr.) Wint. On *Fagus grandifolia*, Upper Brookside, July 1, 1931 (351).

The perithecia of this specimen are small, $200\text{--}250 \mu$ in diameter, and erumpent in small clusters through pustulate ruptures of the bark surface. The spores are three-septate, constricted at the central septum, with a longitudinal septum dividing the two central cells, $12.5\text{--}15 \times 5.5\text{--}6 \mu$; the lower half of the spore is more pointed than the upper rounded half. The fungus is somewhat intermediate between *Strickeria*, *Pleospora*, and *Cucurbitaria*, but seems to fit this genus and species best.

Trematosphaeria callicarpa Sacc. On decayed wood, Folley Lake, June 21, 1926 (P380).

The very large, eight-celled brown spores ($75\text{--}89 \times 14\text{--}16 \mu$), with the cells from the central septum toward the ends becoming successively smaller, until the apical cells are small, cap-like, and pale, are characteristic of the species.

Trematosphaeria faginea Morg. On *Fagus grandifolia*, Salmon River, Sept. 7, 1931 (1480); Upper Brookside, June 27, 1933 (1564).

The spores of this species are one-celled, brown, and four-guttulate at first, but become three-septate at maturity. The barely immersed perithecia appear as thickly scattered, blackened, hemispheric pustules on the bark surface.

Ceratostomeae

Ceratostoma parasiticum E. and E. On *Fomes fomentarius* (L.) Gill., Folley Lake July 20, 1931 (1106).

Mycosphaerellaceae

Mycosphaerella colorata (Pk.) Earle. On leaves of *Kalmia angustifolia* L., Upper Brookside, July 22, 1929 (105); Portapique Beach, Aug. 7, 1933 (1617).

The spots caused by this fungus are at first red-brown with darker raised margins, as given for *M. colorata*. Later the centres bearing the perithecia are dead and grayish. The spores of these collections were $11.5\text{--}14.5 \times 2 \mu$ and somewhat curved. The spores of *M. colorata* are given as $14\text{--}18 \times 2.5 \mu$. *Sphaerella brachytheca* Cke., on *Vaccinium vitis-idaea* L., has similar spots and smaller spores ($14\text{--}15 \times 3 \mu$). There are other species of *Sphaerella* described on Ericaceae which are very similar. Ellis's N.A.F. No. 899, of *Sphaerella colorata* Pk., has identically formed spots but only immature asci could be found in this collection.

Mycosphaerella Coptis (Schw.) House. On leaves of *Coptis groenlandica* (Oeder) Fern., Moore's Lake, Halifax Co., July 6, 1929 (32).

The spores of this collection are immature, $9\text{--}12.5 \times 2.5 \mu$, and still one-celled and four-guttulate, but apparently will become two-celled. The material agrees well with Ellis's N.A.F. Nos. 2358 and 2359 of this species.

This collection is accompanied by a species of *Septoria* with spheric pycnidia, $100\text{--}150 \mu$ in diameter, sunken in the circular grayish spots which are surrounded by a purplish-black discoloration. The conidia are acicular, $40\text{--}71 \times 0.5\text{--}1 \mu$. This is probably *Septoria Coptidis* B. and C., although Berkeley gives the spots caused by his species as rufous with a red-brown margin and the spores as 25μ long.

Mycosphaerella punctiformis (Pers.) Schroet. On leaves of *Fagus grandifolia*, Deep Hollow, Wolfville, June 25, 1926 (P372), mostly sterile material.

Mycosphaerella Sarraceniae (Schw.) House. On leaves of *Sarracenia purpurea* L., Castle Rey Lake, Aug. 10, 1933 (1626).

Mycosphaerella Virgaureae Krieg. On stems of *Solidago* sp., Upper Brookside, June 19, 1933 (1522).

Of the species of *Mycosphaerella* on *Solidago*, *Sphaerella nebulosa* (Pers.) on stems is described as having a superficial appearance identical with this collection, but the spores ($14\text{--}16 \times 5 \mu$) and asci ($50 \times 15 \mu$) are larger. *M. Virgaureae* has a similar appearance on leaves with asci $27\text{--}30 \times 5 \mu$ and spores $10\text{--}15 \times 2\text{--}3 \mu$. The asci of this collection are $26\text{--}35 \times 7\text{--}9 \mu$ and the spores $10\text{--}11 \times 2.5\text{--}3 \mu$.

Phaeosphaerella pheidasca (Schroet.) Sacc. On *Juncus* sp., Victoria Park, June 26, 1935 (1706).

Pleosporaceae

Didymella tosta (Berk. and Br.) Sacc. On *Epilobium angustifolium* L., Victoria Park, July 18, 1933 (1589).

Petrak (14, p. 237) has made this species the type of a new genus, *Paradidymella*, which includes those species of *Didymella* with a sphaeriaceous type of perithecium.

Didymosphaeria populifolia E. and E. On leaves of *Populus tremuloides* Michx., Salmon River, July 7, 1933 (1579).

This collection agrees with the type (Dearnness No. 2263, 1894) of this species, kindly sent by Dr. Dearnness. This may be a synonym of *Phaeosphaerella macularis* (Fr.) Trav., for the asci are fasciculate and without paraphyses.

Didymosphaeria Thalictri Ell. and Dearn. On *Thalictrum* sp., Folleigh Lake, June 21, 1926 (P366); Victoria Park, June 26, 1933 (1556).

Leptosphaeria

This genus is a most interesting one. Many of the species show minor differences of spore structure, often correlated with the host. In the descriptive literature, these details are not always given. Large numbers of species have been described, largely on host distinctions, on the one hand, whereas many host varieties have been obscured by inclusion in one of the ubiquitous species on the other hand. As a result it is difficult to be sure of species determinations without a comparative study of the entire genus. In order to facilitate such comparisons, descriptions of the spores are given for the collections here discussed.

Leptosphaeria anisomeres sp. nov. (Figs. 3 to 5).

Appearing on the surface as effuse, splotched, or blackened areas of the leaf sheath, through which the minute papillate ostioles are erumpent, each through a minute swelling caused by the immersed perithecium, but scarcely visible without a strong lens. Perithia clustered or densely crowded, $150-200 \times 100 \mu$, formed beneath the epidermis and surrounded by a proliferation of dark brown hyphae which cause the blackening of the surface. Walls rather thin and equal in thickness. Asci clavate with a tapering base and a slightly thickened apical wall, $50-60 \times 6-7 \mu$. Spores biseriata above, uniseriate below, fusoid, inequilateral to slightly curved, three- to five-septate, not constricted at the septa, yellow-brown, $12.5-14.5 \times 2.5-3.5 \mu$. The young spore is four-celled with the upper two cells ($6-6.5 \mu$) shorter than the lower ($7.5-8 \mu$). Either one of the larger (lower) cells, or both, may form a second septum.

Type: Herbarium L. E. Wehmeyer, on *Agropyron repens*, Upper Brookside, Colchester Co., N.S., 22, VI, 1933, leg. L. E. Wehmeyer (No. 83).

The proliferation of dark hyphae about the perithecia resembles that found in *Kalmusia*, but no species with small spores and this peculiar type of septation could be found in either genus on Gramineae.

Leptosphaeria anisomeres sp. nov. Superficialiter effusa, maculosa, nigrescens, obvia in foliorum vaginis *Agropyronis repentis*; ostioliis minutis (solum sub lente vix visibilibus), papilliformibus erumpentibus ab areolis minutis elevatis circumdatis; peritheciis immersis, plus minusve dense aggregatis, diametro $150-200 \mu$, altitudine 100μ , subepidermalibus et circumdatis a hyphis atrobrunneis superficiem vaginae nigricantibus; membranis tenuisculis equalibus; ascis clavatis, basi angustatis, apice membrana subincrassata praeditis, $50-60$ longis, $6-7 \mu$ crassis; sporis in parte ascorum superiore biseriatis, in parte inferiore uniseriatis, fusoides, inaequilateralibus vel paulum curvatis, 3- vel 5-septatis, ad septa haud constrictis, luteo-brunneis, $12.5-14.5 \mu$ longis, $2.5-3.5 \mu$ crassis, juvenate saepissime 4-cellulis, cellulis 2 superioribus quam 2 inferioribus brevioribus, superioribus nunquam dividitibus, aetate interdum 5- vel 6-cellulis a divisione cellularum inferioribus singulis vel ambabus.

Specimen typicum in auctoris herbario conservatum, legit L. E. Wehmeyer (No. 1683) in loco dicto "Upper Brookside, Colchester Co." in Nova Scotia, 22, VI, 1933.

Proliferatione hypharum nigrescentium circum perithecia *Kalmusiae* similis, sed a speciebus omnibus *Kalmusiae* et *Leptosphaeriae* gramina instantibus differt sporis parvis inaequaliter septatis.

Leptosphaeria Coniothyrium (Fck.) Sacc. On *Rubus* sp., Victoria Park, July 6, 1931 (407); Upper Brookside, Aug. 11, 1931 (463).

Associated with *Pyrenopeziza Rubi* (Fr.) Rehm. Spores fusoid, four-celled, inequilateral or curved, constricted at the central-septum and sometimes less so at the other septa, end cells somewhat longer than the central cells, $12-16 \times 3-4 \mu$. This collection differs from those placed under *L. dumetorum* chiefly in the shorter spores and crowded perithecia, causing a blackening of the stem surface.

Leptosphaeria Dollolum (Pers.) Ces. and de Not. On *Solidago* spp., Upper Brookside, Aug. 1, 1929 (207); Victoria Park, Aug. 3, 1929 (267); Salmon River, Aug. 1, 1931 (1208); Aug. 1933 (1650); on *Aster* spp., Upper Brookside, July 15, 1929 (74); Victoria Park, June 26, 1933 (1559).

This most widespread species has the thick, "stromatic", perithecial wall typical of the Pseudosphaeriaceae. The asci are rather long-cylindric, $75-90 \times 6-7 \mu$, and the spores are characteristically fusoid, slightly curved or lunate, four-celled, rather dark brown, not constricted at the septa, or very slightly so in age, $17-25 \times 4.5-5 \mu$.

Leptosphaeria dolioloides (Auersw.) Karst. On *Tanacetum* sp., June 26, 1933 (1541).

The spores of this collection are long-fusoid, pale greenish-yellow, slightly curved, nine-celled, not constricted at the septa but with the fourth cell swollen, and $40-44 \times 4-4.5 \mu$. These spores are more constant in their septation and greater in diameter than given for this species, which may be a mixture of varieties.

Leptosphaeria Dumetorum Niessl. On *Clematis* sp., Salmon River, Aug. 1, 1931 (1207); on *Sambucus pubens*, June 27, 1935 (1708).

Under this species name are placed two collections with four-celled, fusoid, yellow-brown spores, $16-19.5 \times 3.5-4.5 \mu$ which have the second cell slightly enlarged. The perithecia are more globose and thinner-walled than the "stromatic" type of *L. Dollolum* and the spores are more constantly constricted and at the lower end of the range of measurements for that species.

The collection on *Clematis* has thickly scattered globose perithecia with rather prominent conic ostioles. The spores are inequilateral or somewhat curved, constricted at the central septum, and with the second cell only occasionally or very slightly swollen. Most species reported on *Clematis* have much larger spores. *L. pyrenopezizoides* Sacc. and Speg. with similar spores differs in the smaller perithecia ($\frac{1}{8}$ mm.) which collapse pezizoid.

On *Sambucus*, the perithecia are more scattered with less prominent ostioles. The spores are old, more strongly constricted at all septa, almost straight, and with a more prominent second cell. There are occasional small spores which are without constrictions and which measure $12-17 \times 3.5-5.5 \mu$.

Leptosphaeria Ellisiana Berl. On *Oenothera* sp., Portapique Beach, Aug. 3, 1933 (1609).

The spores of this collection are fusoid, somewhat curved, four-celled, strongly constricted at the central septum, constricted at the remaining septa when fully mature, and $28-35 \times 5-6 \mu$. They are characterized by a slight asymmetry in which one-half of the spore is somewhat broader and more rounded at the tip and the other half is slightly narrower and more tapered.

Leptosphaeria Onagrae Rehm. is this same species. Rehm. Asc. No. 2080 of *L. Onagrae*, on *Onagra* (*Oenothera*) *strigosa* (Rydb.) Mack. and Bush, has spores which are slightly smaller ($26.5-31 \times 4.5-5 \mu$) and show a more pronounced asymmetry than spores of the type collection (Ellis's N.A.F. No. 697 of *Sphaeria subconica*) of *L. Ellisiana*. Unless this is correlated with the host, however, it lies within the specific range of variation.

Leptosphaeria herpotrichoides de Not. On *Poa* sp., Victoria Park, June 23, 1926 (P363); on *Agropyron repens*, Upper Brookside, June 22, 1933 (1670 and 1683).

These three collections on grass culms have similar cylindric-fusoid spores which are six- to seven-septate and have the third cell somewhat swollen. The collections on *Agropyron* have spores somewhat shorter ($19.5-26.5 \times 3.5-4.5 \mu$) than those found on *Poa* ($24-28-35 \times 4-5 \mu$) which are more variable. This fungus on *Agropyron* might be considered as *L. culmifraga* var. *bromicola* Bres.

Leptosphaeria Kalmiae Pk. On *Kalmia angustifolia*, Portapique Beach, Aug. 7, 1933 (1616 and 1621).

The spores of these collections approach the *Melanomma* type. They are fusoid-ellipsoid, yellow-brown, four-celled, straight or slightly curved, constricted at the central septum and later at all three septa, $13-17 \times 4.5-5 \mu$. The spores are two-celled at first and one-half of the spore is often broadly rounded, with the other half more tapered.

Leptosphaeria Millefolii (Fck.) Niessl. On *Achillea millefolium* L., Upper Brookside, June 23, 1933 (1532).

The spores of this collection are long-fusoid, yellow-brown, eight-celled, tapered equally toward either end, constricted at the central septum, and with the cell on either side of the constriction definitely enlarged. They measure $34-44 \times 4-5 \mu$.

Leptosphaeria ogilviensis (B. and Br.) Ces. and de Not. On *Solidago* sp., Upper Brookside, June 18, 1926 (P361); on *Chrysanthemum* sp., Victoria Park, Truro, June 26, 1933 (1557).

Various species of *Leptosphaeria* may be found in the exsiccata under this binomial. The one most generally found and fitting most descriptions, as far as they go, has a very characteristic spore. These spores are long-fusoid, slightly curved, light yellow-brown, six-celled, somewhat broader and constricted at the central septum and $30-38 \times 3.5-4.5 \mu$. One end of the spore tapers gradually to a narrow tip; the other has a rounded apex, is somewhat narrower in diameter just below this apex, and widens again to a greater diameter in the region of the central septum. The spores of the collection on *Chrysanthemum* are of this type. Those on *Solidago* are smaller ($25-32 \times 2.5-3.5 \mu$) and do not show this asymmetry. These may be merely immature spores, but such spore differences are often constant in this genus.

Leptosphaeria planiuscula (Riess) Ces. and de Not. On *Solidago* spp., Onslow Marsh, Truro, June 19, 1926 (P365); June 19, 1933 (1519 and 1520); Portapique Beach, June 31, 1933 (1604); Victoria Park, June 26, 1933 (1654); on *Aster* sp., Upper Brookside, July 27, 1931 (1160 and 1161).

These collections have spores which are fusoid, yellow-brown, straight or slightly curved, symmetrical, four-celled at first, becoming six-celled, constricted at the central septum at first, finally constricted at all the septa, $50-67 \times 7-9 \mu$. The spores often show faint cap-like protoplasmic appendages at the ends.

Leptosphaeria rhopalspora Berl. On *Gnaphalium* sp., Victoria Park, June 26, 1933 (1542); Salmon River, July 7, 1933 (1574).

The spores of these collections are clavate-fusoid, rounded at one end and tapered toward the other, yellow-brown, slightly curved, four-celled at first, then six-celled, $25-33 \times 4-5 \mu$, and constricted at the septa only at full maturity. They fit the description and Berlese's figures of this species on *Inula*, but no material has been seen for comparison.

Leptosphaeria vagabunda Sacc. On *Solidago* sp., June 19, 1933 (1520).

L. Dolium occurs on these same stems, but the perithecia of this species are more globose and thinner walled and the asci ($82-90 \times 14-16 \mu$) are more broadly clavate. The spores are more crowded, biserial, ellipsoid, straight or slightly curved, four-celled, more strongly constricted at the septa, often with the second cell slightly enlarged, and measure $21-26 \times 7-8.5 \mu$.

Ophiobolus acuminatus (Fr.) Duby. On *Cirsium* sp., Victoria Park, June 26, 1933 (1544); Salmon River, July 7, 1933 (1575).

The spores of this species are many-celled, $110-135 \times 2-3 \mu$ and with one swollen cell excentrically placed, or with two adjacent cells swollen at the ends opposite the common septum. The spores tend to constrict at the septa and fall apart into biguttulate cells. Berlese (1, p. 88) created the genus *Leptosphaeriopsis* for those species which had 16-spored asci, with the spores attached in pairs, by their ends, and included this species therein. This is merely a roundabout interpretation, of course, of the fact that the spores tend to fall apart at the middle between the two swollen cells.

Ophiobolus porphyrogonous (Tode) Sacc. On *Corallorhiza maculata* Raf., Wolfville, June 25, 1926 (P249b); on *Spiraea* sp., Salmon River, Sept. 3, 1929 (269).

Physalospora Laricis sp. nov. (Figs. 6 to 8).

Perithecia spheric, 400-500 μ in diameter, somewhat flattened or concave on top with a central papillate ostiole, thickly scattered, singly erumpent through the periderm, eventually falling out and leaving a circular perforation, white within, walls 50-80 μ thick, composed of large, dark-brown-walled pseudoparenchyma. Asci large, stout-clavate, with a short stipe and a claw-like base, apical wall strongly thickened, (90) $115-150 \times 26.5-32 \mu$, embedded in an interthecial tissue of paraphysis-like strands. Spores biserial, ellipsoid to fusoid-ellipsoid or inequilateral, one-celled, brown, often with a disk-like refractive body in the centre of the spore, $34-41 \times 12-14 \mu$.

Type: Herbarium L. E. Wehmeyer, on *Larix laricina*, Upper Brookside, Colchester Co., N.S., 8, VII, 1931, leg. L. E. Wehmeyer (No. 434); on *Larix*, Salmon River, N.S., 3, VII, 1931 (No. 374).

This is a typical *Physalospora*, the perithecia being thick-walled and pseudosphaeriaceous in structure with a small pore-like ostiole, but the spores are definitely brown. Theissen would use this genus name for *sphaeriaceous* forms, whereas Höhnelt uses it for the pseudosphaeriaceous ones. The original generic description allows the inclusion of coloured spores. It seems strange that this striking fungus has not been described, but no fitting description could be found in any likely genera. It does not have the clypeus

of *Anthostomella*, and *Paranthostomella*, without a clypeus, contains no similar species. The genus *Maurinia* is supposed to have asci with a plug in the ascus tip which stains blue in potassium iodide, which is not true of this collection. *Phaeobotryon* (*Phaeobotryosphaeria*) has compound stromata.

Physalospora Laricis sp. nov. Perithecia sphaerica, 400–500 μ diametro, apice applanata vel concava et ostiolo centrali papilliformo praedita, densiuscule dispersa, singula per peridermate erumpentia, intus alba, demum delapsu perforationem circularem relinquuntia; parietibus 50–80 μ crassis, parenchymatis, atrobrunneis, grandicellulis; ascis magnis, breviter clavatis, breviter stipitatis, basi deflexis, apice valde incrassatis, 115–150 μ longis, 26.5–32 μ crassis, inter filamenta interthecialia paraphysiformia immersis; sporis biseriatis, ellipsoideis vel fusiformibus vel inaequilateralibus, 1-cellulis, bruneis, saepe somate disciformi refractivo centrali praeditis, 34–41 μ longis, 12–14 μ crassis.

Specimen typicum in auctoris herbario conservatum, in ramis ramulisque *Laricis laricinae* prope locum dictum "Upper Brookside, Colchester Co." in Nova Scotia, legit L. E. Wehmeyer (No. 434), 8, VII, 1931; etiam in *Larice*, Wehmeyer No. 374, ad "Salmon River" in Nova Scotia, 3, VII, 1931.

Pleospora herbarum (Pers.) Rab. On *Lathyrus japonicus* Willd., Portapique Beach, Aug. 3, 1933 (1672).

Pleospora nitida (E. and E.) comb. nov. (*Teichospora nitida* E. and E. Proc. Acad. Natural Sci. Phila.). On *Rubus* sp., Wolfville, June 25, 1926 (P359).

This collection fits the description of *Teichospora nitida* E. and E. and agrees with the Nuttall collection (Fl. Fayette Co., W. Va. No. 1817) cited by Ellis. As stated in the description, however, the perithecia are formed beneath the periderm and exposed by the exfoliation of this tissue. It belongs properly in the genus *Pleospora*.

Venturia curviseta Pk. On leaves of *Nemopanthus mucronatus* (L.) Trel., Grande Anse, Richmond Co., Aug. 3, 1931 (1236).

The spores of this collection are brown when fully mature, and $8.5\text{--}11 \times 4\text{--}5.5 \mu$. The asci are cylindric, 53–60 \times 5–7 μ . The perithecia are 100–150 μ in diameter and have the typical recurved setae.

Venturia Gaultheriae E. and E. On leaves of *Gaultheria procumbens* L., Upper Brookside, July 14, 1929 (48).

Venturia inaequalis (Cke.) Wint. On leaves of *Malus pumila* Mill., Kentville, Kings Co., March 27, 1927 (116), perithecia immature.

Gnomoniaceae

Apiognomonina guttulata (Starb.) comb. nov. (*Gnomoniella guttulata* Starb. Ascom. Oeland, p. 10). On *Agrimonia* sp., Victoria Park, Truro, Sept. 7, 1929 (291); June 23, 1933 (1538).

The original description gives the spores of this species as cylindric-fusoid, inequilateral to curved, narrowed downward, with a spurious septum below the middle, $6\text{--}10 \times 1\text{--}2.5 \mu$. This septum is usually present and visible and the lower cell is usually smaller than the upper. The species, should be, therefore, in the genus *Apiognomonina*.

Gnomonia rostellata (Fr.) Wehm. On *Rubus* sp., Upper Brookside, July 21, 1929 (109).

As pointed out by the writer (19, p. 266), this species should be in the genus *Gnomonia* rather than *Diaporthe*. *G. Rubi* Rehm. is probably the same species on leaves.

Cucurbitariaceae

Gibberidea abundans (Dobr.) Shear (*Naumovia abundans* Dobr.). On *Lycopus americanus* Muhl., Wolfville, June 26, 1926 (P373); on *Prunella vulgaris* L., Upper Brookside, July 4, 1931 (1400).

Shear (17, p. 358) is correct in all he says concerning the dothideaceous character of this fungus, and its close relationship to *Rosenscheldia* Speg. and *Gibberidea* Fck. The general citation of the spores of these genera as many-celled and brown, without qualifications, is misleading, inasmuch as the spores are commonly immature, one-celled, and hyaline when collected and become definitely septate and pale brown only at full maturity. The spores of these collections were one-celled, hyaline, and $26\text{--}30 \times 1.5 \mu$ in No. 400 and hyaline to pale brown, one-celled, and $33\text{--}39 \times 1.5\text{--}2 \mu$ in No. P373.

Probably all of the Cucurbitariaceae and numerous other forms now placed in the Sphaeriales have what is considered a dothideaceous structure. This species is retained in this family for the present.

Gibberidea alnea (Pk.) comb. nov. (*Cucurbitaria alnea* Pk. Rept. New York State Museum, 28: 75. 1878.). On *Alnus* sp., Green Oaks, Colchester Co., July 12, 1929 (180).

Appearing on the surface as clusters of spheric, brown-black perithecia, confluent or seated on a subdothideaceous stroma erumpent through lateral ruptures of the periderm. The perithecia are 300–400 μ in diameter, and in sections whitish within. Asci long-clavate with a tapering base, spore-bearing portion 85–100 \times 8.5–10 μ ; stalks 15–25 μ . The spores are irregularly biseriata, fusoid-ellipsoid, hyaline, four-guttulate at first, then two-celled with a swelling above the septum, and finally four-celled, with the second cell enlarged and a large guttule in each cell, eventually pale brown at maturity, 20–25 \times 5–7 μ . When fresh the spores often show short, hyaline, apical appendages containing two refractive granules. Paraphyses numerous, hyaline, filiform, persistent.

Peck (12, p. 75) described his *Cucurbitaria alnea* as having uniseriate, uniseptate, hyaline, two- to four-guttulate spores, 20–25 μ long. It seems probable, however, that he had a young stage of this same fungus. The structure of this material is the same as that of the preceding species, except for the somewhat broader, more definitely septate and constricted spores, which are of the same type as those of *Pseudotrichia aurata* and many other fungi scattered throughout the Sphaeriales. It should be placed in the genus *Gibberidea* as outlined by Shear. *Gibberidea alnicola* Rehm. has smaller spores (12–15 \times 4.5–5 μ).

Massariaceae

Massaria inquinans (Tode) Ces. and de Not. On *Acer spicatum*, Upper Brookside, July 4, 1931 (395); Sept. 4, 1931 (1685).

Massaria pruni Wehm. (*M. occulta* (Schw.) E. and E.). On *Betula papyrifera* Marsh., Upper Brookside, July 13, 1931 (500); on *Prunus* (?), July 13, 1931 (1007); on *Amelanchier*, Portapique Beach, July 26, 1933 (1597).

This name change was made by the writer (21, p. 131) because of the pre-existing *Massaria occulta* Romell. There is a legitimate doubt as to the hosts of these collections and they may all be on *Amelanchier*. The spores of these collections are hyaline at first but soon turn brown; they measure 58–64 \times 14 μ in No. 500; 60–69 \times 14–16 μ in No. 1007 and 63–71 \times 13–14.5 μ in No. 1597. The type collection, on *Prunus*, has somewhat smaller spores (52–60 \times 12–13 μ) which were all hyaline (although Ellis states that the spores turn brown). It may be that this is a variety on *Amelanchier*, as the species of *Massaria* show minor differences correlated with the host, but a comparative study of more material on these hosts must first be undertaken.

Massaria (Massarina) salilliformis sp. nov. (Fig. 9 and Fig. 10).

This appears on the surface as thickly scattered blackened areas, 2–5 mm. in diameter, each of which consists of a crowded group of minute papillate pustules which are soon perforated by the black punctate mouths of the separately erumpent ostioles. Perithecia 300–400 \times 250 μ , flask-shaped, with thick (30–40 μ) black walls, immersed in the unaltered bark and erumpent directly through the surface by a short ostiolar neck. Asci stout-clavate with a thickened apical wall, (100) 170–210 \times 23 μ , embedded in a mass of filiform, hyaline, persistent paraphyses. Spores biseriata, cylindric-fusoid, hyaline, ends blunt, 6- to 10-celled or guttulate, not constricted at the septa, (35) 40–47 \times 10–12 μ .

Type: Herbarium L. E. Wehmeyer, on *Fagus grandifolia* bark, Salmon River, Colchester Co., N.S., 7, IX, 1931, leg. L. E. Wehmeyer (No. 1684); on living bark of *Fagus*, Upper Brookside, 1, VII, 1931 (No. 350).

This species is placed provisionally in the genus *Massaria*. The entire family of the Massariaceae needs revision badly, as it contains a number of distinct groups related to scattered species in many genera. This collection belongs to a group typified by long cylindric-fusoid, many-celled, hyaline, non-constricted spores. It is difficult to say where it might have been described previously, but no similar species could be found in *Massaria*, *Massarina*, *Metasphaeria*, or *Calospora*.

Massaria (Massarina) salilliformis sp. nov. In areis superficialibus *Fagi* ramulorum nigrescentibus densis 2–5 mm. diametro, pustulae minute papillatae dense aggregatae mox perforatae a ostiolis nigris separate erumpentibus; peritheciis 300–400 μ altis, 250 μ crassis, ampulliformibus pariete 30–40 μ crasso, nigris, in cortice sano immersis et per orem brevem singulis erumpentibus; ectostromate nullo; ascis breviter clavatis apice incrassatis 170–210 μ longis, ca. 23 μ latis, inter paraphyses persistentes hyalinos filiformes immersis; sporis biseriatis, cylindric fusiformibus, hyalinis, utrinque obtusis, 6–10-cellulis vel guttulatis, non-constrictis, (35) 40–47 μ longis, 10–12 μ crassis.

Specimen typicum conservatum in auctoris herbario, legit L. E. Wehmeyer (No. 1684), 7, IX, 1931. Habitat in cortice *Fagi grandifoliae* ad "Salmon River, Colchester Co." in Nova Scotia; "Upper Brookside", Nova Scotia (Wehmeyer No. 350) in cortice *Fagi* viventis.

Allantosphaeriaceae

Diatrype Stigma (Hoffm.) Fr. On *Betula papyrifera*, Upper Brookside, July 8, 1931 (431), common.

Diatrypella

The species of *Diatrypella* are difficult to separate. The character of the stroma has been used to separate species, but this is affected by the type of bark and the manner of growth. Ascus size, although probably a diagnostic character, is affected by the variable length of the stalk, the variable distribution of the spores in different asci of different age, when the spore-bearing portion is measured, and the often overlooked empty tip of the ascus. The diameter of the ascus is probably the most dependable measurement. The spores of different species (?) have such overlapping ranges that these must also be used with caution. Perhaps the clearest way to present the facts of these collections is to tabulate the characters of the relevant species and those of the collections concerned. The ascus measurements are taken from the tip to the narrowed basal stalk (spore-bearing portion).

Collection	Stroma	Measurements, μ		Host
		Asci	Spores	
<i>D. decorata</i>	Ovoid	40-48 \times 5	5-6 \times 1	<i>Betula</i>
<i>D. discoidea</i>	Ovoid to discoid	Not given	5 \times 0.75-1	<i>Betula</i>
<i>D. favacea</i>	Ovoid	70-100 \times 9-12	6-8 \times 1.5	<i>Betula</i>
<i>D. Tocciaana</i>	Angular discoid	100-120 \times 12	5-7 \times 1	<i>Betula</i> and <i>Alnus</i>
<i>D. nigro-annulata</i>	Angular discoid	100-180 \times 10-12	6-8 \times 1.5	<i>Fagus</i>
<i>D. Demetronis</i>	Discoid	35 \times 6	4-5 \times 1-1.5	<i>Salix</i>
No. 203	Discoid	37-45 \times 5-5.5	3.5-4.3 \times 0.6-0.8	<i>Prunus</i>
No. 104	Discoid	30-40 \times 4-6	3.5-5 \times 0.8	<i>Betula</i>
No. 477	Discoid	35-43 \times 5-5.5	4.3-5 \times 0.5-0.8	<i>Betula</i>
No. 286	Angular discoid	80-110 \times 8.5-10	4.5-6 \times 1-1.2	<i>Alnus</i>
No. 1463	Angular discoid	88-125 \times 7-9	5-6 \times 1	<i>Fagus</i>
No. 397	Ovoid	Not seen	5-6 \times 1-1.5	<i>Betula</i>
No. 1675	Ovoid	60-70 \times 7-9	4-6 \times 1-1.5	<i>Betula</i>
No. 1154	Ovoid	50-70 \times 11-12	4-6 \times 1	<i>Betula</i>

From these data, these collections might be distributed as given below. Whether these are all good species is another question.

Diatrypella betulina Pk. On *Betula papyrifera* and *Betula* spp., Upper Brookside, July 13, 1926 (98 and 106); Princeport, July 9, 1931 (469); Victoria Park, Aug. 15, 1933 (1632).

This species is distinguished by the greenish colour of the interior of the stroma.

Diatrypella discoidea Cke. and Pk. On *Betula* sp., Westcooke's Grove, Guysboro Co., Sept. 7, 1925 (104); Upper Brookside, July 10, 1931 (477); on *Prunus* sp., Upper Brookside, July 30, 1929 (203).

This group of collections with short and narrow asci, small, narrow spores (less than 1 μ in diameter) and small discoid stromata, are arbitrarily placed in *D. discoidea*. In his description, Peck (12, p. 71) merely says "asci small." *D. Demetronis* E. and E. fits these collections very well except for the greater diameter of the spores. *D. decorata* Nit. of Europe is also similar, but with larger spores and laterally elongate stromata.

Diatrypella favacea (Fr.) Nit. On *Betula* spp., Wolfville, June 25, 1926 (397); New Glasgow Rd., July 25, 1931 (1154); Victoria Park, Aug. 15, 1933 (1675).

These collections have the characteristic, laterally elongate stromata of this species. The spores of these collections, and of most other American collections, are somewhat smaller than the measurements given for this species in Europe, and copied in American descriptions.

Diatrypella nigro-annulata (Grev.) Nit. On *Fagus grandifolia*, Upper Brookside, Sept. 3, 1931 (1463).

This and the following species have small angular pustules with closely adherent periderm. The asci are longer than in *D. favacea* and the spores are about the same size. This collection, on *Fagus*, has punctate ostioles rather than the sulcate type usually found in this genus.

Diatrypella Tocciaeana de Not. On *Alnus* sp., Victoria Park, Sept. 7, 1929 (286).

This collection differs from the previous one chiefly in the host and the sulcate ostioles.

Eutypa milliaria (Fr.) Sacc. On wood surface of *Acer* sp., Salmon River, Sept. 2, 1931 (1458); and of *Cornus alternifolia* L., Upper Brookside, July 30, 1929 (152).

The spores of these collections ($5-7 \times 1.5 \mu$) are somewhat smaller than those given for this species. Both show the blackened surface crust. Conidiophores, but no conidia, were present on *Acer*. On *Cornus*, lunate-fusoid, filiform, hyaline, one-celled conidia $16-23 \times 0.8-1 \mu$ were found in irregular cavities beneath the blackened surface.

Eutypa spinosa (Pers.) Tul. On *Fagus grandifolia*, Upper Brookside, Aug. 12, 1931 (1284) leg. A. H. Smith.

Eutypella alnifraga (Wahl.) Sacc. On *Alnus* sp., Salmon River, Aug. 1, 1931 (1211); Victoria Park, Aug. 13, 1935 (1781).

Diaporthaceae

Apioportha anomala (Pk.) Höhn. On *Corylus cornuta* Marsh., Upper Brookside, July 14, 1929 (65).

Apparently parasitic and killing the canes which are then covered with the strongly pustulate stromata of this fungus.

Apioportha Corni Wehm. On *Cornus alternifolia*, Upper Brookside, July 30, 1939 (148); Victoria Park, June 26, 1935 (1711).

Causing a characteristic orange discoloration of the dead limbs of this host. Both collections are accompanied by the conidial stage, *Zythia aurantiaca* (Pk.) Sacc.

Apioportha phomaspora (Cke. and Ell.) Wehm. On *Myrica pennsylvanica* Lois., Evangeline Beach, Wolfville, June 26, 1926 (P369).

Apioportha vepris (DeLacr.) Wehm. On *Rubus* sp., Victoria Park, June 23, 1926 (P148b).

Cryptodiaportha galeculata (Tul.) Wehm. On *Fagus grandifolia*, Upper Brookside, July 21, 1929 (104).

Cryptodiaportha salicina (Curr.) Wehm. On *Salix* sp., Onslow Marsh, Truro, June 19, 1933 (1521).

This is associated with the conidial stage, *Discella carbonacea* (Fr.) Berk. and Br.

Cryptospora alnicola Höhn. On *Alnus* spp., Salmon River Marsh, Aug. 3, 1929 (279); Upper Brookside, July 13, 1931 (1005); Portapique Beach, Aug. 6, 1935 (1767).

The species of *Cryptospora* are badly in need of revision. Aside from *C. femoralis* Pk., which is a clear-cut species, the collections here considered on *Alnus* and *Corylus* all have spores and asci which are practically identical in their range of size and structure. These spores are long-cylindric, flexuous, usually non-septate and many-guttulate, but finally showing faint septa. They range in length from $50-88 \mu$ but are comparatively narrow ($2-3.5 \mu$). The collections placed under *C. alnicola* have small but sometimes rather strongly pustulate stromata with a closely adherent periderm. There is nearly always a definite, although sometimes minute, grayish ectostroma and often a grayish entostroma about the perithecia which lie in the upper bark and are often bounded below by a blackened zone of bark tissue. The asci are $50-100 \times 7-12 \mu$ and the spores $50-88 \times 2-3.5 \mu$.

The European *C. suffusa* (Fr.) Tul. differs from these American collections on *Alnus* in the broader spores ($45-80 \times 3.5-5 \mu$) and the tendency to form polysporous asci of the "*Diopella*" type, with ellipsoid spores. The above American collections agree with the type of Höhnel's (8, p. 107) *C. alnicola* which was kindly loaned to the writer by Doctor Linder. Höhnel gives the spores as 5- to 10-septate but no septa were seen in the type. Occasional, very faint septa are seen in these collections. The spores of the type are $60-80 \times 2-3 \mu$.

Cryptospora aurantiaca sp. nov. (Figs. 11 to 13).

Stromata forming angular rather prominent pustules, $0.8-1.2$ mm. in diameter, with a central radiate rupture of the closely adherent periderm which exposes a dark brown disk through which three to eight minute, short, spine-like ostioles are erumpent. Perithecia $200-300 \mu$ in diameter, crowded in the bark just beneath the periderm and erumpent through a usually well developed, orange-brown ectostroma. Asci stout-cylindric, $88-95 \times 9-12.5 \mu$. Spores parallel or interwoven in the ascus, long-cylindric, non-septate, many-guttulate, curved, $65-88 \times 2-3.5 \mu$.

Type: Herbarium L. E. Wehmeyer, on *Alnus* sp., Portapique, N.S., 9, VII, 1933, leg. L. E. Wehmeyer (No. 1624).

This species is distinguished by the orange-brown ectostromatic tissue which turns bright wine-red in potassium hydroxide. This colour may be paler in younger stromata. *C. suffusa* (Fr.) Tul. and *C. corylina* (Tul.) Fck. are both given as having yellow discolorations of the stroma but the spores of these species are of greater diameter (3.5–5.5 μ).

Cryptospora aurantiaca sp. nov. Stromata angulosa pustuliformia prominentia, 0.8–1.2 mm. diametro, ad centrum radiate erumpentia per peridermatem et ectostroma aurantiaca copiosa; ascis crassis, cylindricis, 88–95 μ longis, 9–12.5 μ crassis; sporis in asco parallelis vel intertextis, longe cylindricis, non-septatis, pluriguttulatis, curvatis, 65–88 μ longis, 2–3.5 μ crassis.

Specimen typicum in auctoris herbario conservatum, legit L. E. Wehmeyer (No. 1624) in *Alni* cortice prope Portapique in Nova Scotia, 9, VII, 1933.

A speciebus aliis differt ectostromate aurantiaco-brunneo kali caustici actione laete vinaceo vel in stromatis juvenilibus pallidiuscule rubro. A *C. suffusa* et *C. corylina* differt discoloratione stromatis aurantiaca vel brunnea haud lutea, et sporis minoribus (sporis in speciebus ambabus aliis 3–5.5 μ diametro).

Cryptospora Betulae Tul. On *Betula papyrifera*, Upper Brookside, July 14, 1929 (61).

Cryptospora femoralis Pk. On *Alnus* spp., Rifle Range, Truro, June 19, 1926 (P367); Salmon River, July 1, 1929 (13); New Glasgow Rd., June 30, 1931 (335); Portapique Rd., July 9, 1933 (1623); Victoria Park, June 27, 1925 (13a).

This very common species has characteristic spores with swollen ends.

Cryptospora suffusa var. **nuda** Pk. On *Corylus cornuta*, Black Rock, Shubenacadie River, June 28, 1929 (17); Victoria Park, June 29, 1935 (1715).

These collections on *Corylus* differ from *C. alnicola* only in the host and in the general lack of any grayish ecto- or entostroma or any blackened zone in the bark, resulting in minute disks of clustered spine-like ostioles. Peck (13, p. 58) gives his variety *nuda* as differing from *C. suffusa* as follows, "Stroma not suffused with a yellow dust . . . the black circumscribing line is also apparently absent in some cases". He gives the variety as on *Alnus* and *Corylus* and apparently includes *C. alnicola*, as here interpreted, in this conception. His varietal name is used here for the form on *Corylus*.

Diaporthe acerina (Pk.) Sacc. On *Acer spicatum*, Economy Lake, Colchester Co., June 16, 1926 (P52c); Wolfville, June 25, 1926 (P52d); Upper Brookside, July 16, 1931 (1069).

Very abundant on this host.

Diaporthe Arctii (Lasch) Nit. On *Aster* spp., Upper Brookside, July 27, 1931 (1161); Portapique Beach, Aug. 13, 1933 (1611); on *Solidago* sp., Victoria Park, Truro, June 23, 1933 (1536); on *Gnaphalium* sp., Salmon River, July 7, 1933 (1574).

The collections on *Aster* and *Solidago* stems show a quite distinct host form which approaches closely to *D. pardalota* (Mont.) Fck. in the elongate stromatic patches of surface blackening, which are limited in size (1–10 \times 0.5–1 mm.) and often sharply margined, but again confluent and indefinite.

On *Gnaphalium* the surface blackening is entirely absent or limited to minute spots or streaks about the short spine-like ostioles. An accompanying *Phomopsis* stage on this host consists of elongate, raised, blackened stromatic patches, 0.5–1.5 mm. long, containing irregular cavular cavities and alpha conidia which were fusoid, one-celled, hyaline, and 8.5–10 \times 1.5–2 μ .

var. **achilleae** (Auersw.) Wehm. On *Achillea Millefolium* L., Upper Brookside, June 22, 1933 (1531).

Diaporthe decedens (Fr.) Fck. On *Corylus cornuta*, Black River Gorge, Wolfville, June 26, 1926 (P308a); Earltown Rd., Aug. 22, 1931 (308b); Upper Brookside, Aug. 28, 1931 (1677).

Occasional blackened zones were seen at the margins of the entostromata in both No. 308b and No. 1677.

Diaporthe eres Nit. (*D. valida* Nit.). On *Myrica pennsylvanica* Baddeck, Victoria Co., Aug. 4, 1931 (1240).

This seems to be the first report of *D. eres* on *Myrica* (*D. valida* Nit.) from North America.

Diaporthe impulsa (Cke. and Pk.) Sacc. On *Sorbus americana*, Green Oaks, Colchester Co., July 12, 1929 (181).

Diaporthe linearis (Nees) Nit. On *Solidago* spp., Upper Brookside, July 30, 1929 (153); Aug. 1, 1929 (209); Portapique Beach, Aug. 3, 1933 (1608); Salmon River, Aug., 1933 (1650).

Diaporthe oxyspora (Pk.) Sacc. On *Ilex verticillata* (L.) Gray, Evangeline Beach, Wolfville, June 26, 1926 (P364).

Diaporthe quadruplex sp. nov. (Figs. 14 to 16).

Appearing on the surface as minute, black, short, stout, conic to cylindric ostioles which may be erumpent singly but usually occur clustered in longitudinal series. Perithecia flattened-spheric, $300-400 \times 200 \mu$, scattered singly or usually crowded in longitudinal series just beneath the surface and causing a slightly elongate, pustulate swelling. The surface of the bark, beneath the periderm, is blackened locally about the ostioles. No ventral zones in bark or wood. Asci stout clavate with a refractive ring in the apex, four-spored, $47-53 \times 10.5-12.5 \mu$. Spores overlapping biseriate, long fusoid-ellipsoid, somewhat curved, two-celled, hyaline, constricted at the septum, four-guttulate, $22.5-27 \times 2.5-5 \mu$.

Type: Herbarium L. E. Wehmeyer, on *Solidago*, Upper Brookside, Colchester Co., N.S., 1, VIII, 1929, leg. L. E. Wehmeyer (No. 207).

This species has the structure of *D. linearis* but has four-spored asci and spores twice as long as in that species. It may represent a four-spored condition of *D. linearis*. The spores are similar to, but larger than those of *D. semiinsculpta*.

Diaporthe quadruplex sp. nov. In caule *Solidaginis* vel *Astri* visibilis ut ostiola minuta, nigra, brevia, crassa, conica vel cylindrica, solitaria vel uniseriatim gregaria; peritheciis oblate sphaericis, $300-400 \mu$ latis, 200μ altis, solitariis vel longitudinaliter, infra superficiem seriatis et tumiditatem paulum elongatam efficientibus; cortice subepidermali circum ostiola nigrescente; zonis infraperithecialibus nullis vel in cortice vel in ligno; ascis crassis, clavatis, apice annulo refringenti praeditis, 4-sporis, $47-53 \mu$ longis, $10-12.5 \mu$ crassis; sporis biseriatis inter se obtangentibus; elongate fusiformibus vel ellipsoideis, paulum curvatis, 2-cellulis, hyalinis, ad septum constrictis, 4-guttulatis, $22.5-27 \mu$ longis, $3.5-5 \mu$ crassis.

Specimen typicum in auctoris herbario legit L. E. Wehmeyer (No. 207) prope "Upper Brookside, Colchester Co." in Nova Scotia, 1, VIII, 1929.

Diaporthe racemula (Cke. and Pk.) Sacc. On *Epilobium angustifolium*, Deep Hollow Rd., Wolfville, June 25, 1926 (P374); on *Epilobium* spp., Upper Brookside, July 13, 1931 (1012); Victoria Park, Truro, July 18, 1933 (1588).

Diaporthe tessella (Pers.) Rehm. On *Salix* sp., Upper Brookside, June 27, 1931 (306).

Diaporthe tuberculosa (Ell.) Sacc. On *Amelanchier* spp., Portapique Beach, July 26, 1933 (1593); Victoria Park, Truro, June 26, 1935 (1705).

Diaporthe Viburni Dearn. and Bisby var. **spiraecicola** Wehm. On *Spiraea* sp., Salmon River Marsh, Truro, Sept. 3, 1929 (281); Upper Brookside, July 13, 1931 (496).

Melanconis Alni Tul. var. **marginalis** (Pk.) Wehm. On *Alnus* spp., Oldham, Halifax Co., Sept. 5, 1929 (277); Portapique Beach, Aug. 3, 1933 (1607); Mt. Uniacke, June 24, 1935 (1660).

The black conidial masses of the conidial stage of this species (21, p. 27) are common on species of *Alnus* and often accompany the perithecial stage.

Melanconis apocrypta Ell. On *Populus* spp., Salmon River, July 7, 1933 (1578); Upper Brookside, July 9, 1935 (1735).

Melanconis Everhartii Ell. On *Acer spicatum*, Economy River, Colchester Co., Aug. 31, 1927 (35); Earlton Rd., Aug. 22, 1931 (1395).

Melanconis nigrospora (Pk.) Wehm. On *Betula* spp., Victoria Park, Aug. 8, 1929 (219); June 21, 1933 (1527); Aug. 15, 1933 (1630); Upper Brookside, July 3, 1931 (1191); July 9, 1935 (1376).

Melanconis stilbostoma (Fr.) Tul. On *Betula* spp., Upper Brookside, July 8, 1931 (435); July 13, 1931 (1011); Aug. 8, 1931 (429); Victoria Park, Aug. 15, 1933 (1652); Aug. 26, 1935 (1661); Portapique Rd., July 9, 1933 (1622).

This is the commonest pyrenomycete found on down limbs and piled brush of birch. It is commonly accompanied by the blackened masses of the conidial stage, *Melanconium betulinum* Schm. and Kze.

- Melanconis thelebola** (Fr.) Sacc. On *Alnus* spp., Truro, Sept. 18, 1926 (110); Victoria Park, Sept. 7, 1929 (286); Upper Brookside, July 13, 1931 (1671); June 27, 1935 (286a).
Quite common on *Alnus*.
- Pseudovalsa longipes** (Tul.) Sacc. On *Quercus borealis* Michx. var. *maxima* (Marsh) Ashe (*Q. rubra*), Oakfield, Halifax Co., Sept. 8, 1933, leg. A. R. Prince.
- Pseudovalsa stylospora** E. and E. On *Acer spicatum*, Deep Hollow Rd. and Duncan Brook, Wolfville, June 25, 1926 (P371 and P371a); Upper Brookside, July 1, 1933 (1571); Victoria Park, June 29, 1935 (P371b); on *Acer saccharum*, Upper Brookside, July 15, 1929 (46).
- Valsa amphibola** Sacc. On *Sorbus americana*, Victoria Park, Sept. 7, 1929 (288); on *Malus pumila*, Victoria Park, Sept. 7, 1929 (292).
The collection on *Sorbus* is associated with a *Cytospora* with numerous radial locules and conidia which are allantoid, hyaline, $3.5-4.5 \times 1 \mu$.
- Valsa cincta** Fr. On *Amelanchier* sp., Portapique Beach, Aug. 3, 1933 (1606); on *Rosa* sp., Portapique Beach, July 26, 1933 (1598).
The collection on *Rosa* might be considered a variety of this species. It differs from the collection on *Amelanchier* chiefly in the gray rather than brown coloration of the entostroma. *V. leucostoma* var. *Rosarum* Sacc. has smaller spores and smaller disks. These spores on *Rosa* are $12.5-18 \times 2.5-3.5 \mu$.
- Valsa etherialis** E. and E. On *Acer saccharum*, Victoria Park, Aug. 13, 1935 (1780).
- Valsa Kunzei** Fr. On *Abies balsamea*, Moore's Lake, Halifax Co., July 6, 1929 (21); Victoria Park, Aug. 8, 1929 (223); July 8, 1935 (21a); New Glasgow Rd., July 25, 1931 (1676).
Quite common and found on many coniferous genera throughout the United States and Canada. *V. superficialis* Nit. is probably the same species on pine.
- Valsa leucostoma** Fr. On *Prunus* sp., Upper Brookside, July 13, 1931 (1008).
- Valsa nivea** (Hoffm.) Fr. sensu Ellis. On *Populus Tacamahaca* Mill., Victoria Park, Sept. 7, 1929 (290).
This collection has the sharply outlined stromata and minute white disks of this species and the small spores ($7-9 \times 1 \mu$) which Ellis (5, p. 484) says are characteristic of the American form. Spore measurements by European authors are $12-14 \times 3 \mu$. Collections of such a large-spored form (spores $10-18 \times 2-3 \mu$) on *Salix* have been sent from Lake Temagami to the writer by Dr. H. S. Jackson, and apparently exists also on this continent. This small-spored form fits Ellis's description of *V. pallida* very well except for the "subferruginous" disk of that species.
- Valsa salicina** (Pers.) Fr. On *Populus* sp., Upper Brookside, June 28, 1931 (315).
- Valsa sordida** Nit. On *Salix* spp., Evangeline Beach, Wolfville, June 26, 1926 (Herb. No. 3001); Salmon River Marsh, Truro, Sept. 3, 1929 (273); on *Populus* sp., Oct. 2, 1926 (100).
- Valsa stenosporea** Tul. On *Alnus* sp., Oldham, Halifax Co., Sept. 5, 1929 (277).
This collection is characterized by the minute white to grayish disks, closely adherent periderm, pulvinate swellings above the perithecia and spores $9-12 \times 1.7-2.5 \mu$.
- Valsa truncata** Cke. and Pk. On *Alnus crispa* (Ait.) Pursh. var. *mollis* (Fern.) Fern., Black River Gorge, Wolfville, June 25, 1926 (P379); on *Alnus* sp., Salmon River, July 1, 1929 (11); Mount Uniacke, June 24, 1935 (1700).
These collections have truncate-conic stromata with laterally elongate brown to black disks and spores $8.5-11 \times 1-1.5 \mu$.
- Anthostoma melanotes** (B. and Br.) Sacc. On decorticated wood of *Salix* or *Alnus*, Salmon River, Aug. 1, 1931 (1210).
- Fenestella minor** Tul. On *Alnus* sp., Salmon River, July 14, 1931 (1017).
- Valsaria moroides** (Cke. and Pk.) Sacc. On *Alnus* sp., Victoria Park, July 23, 1931 (1124).

Calosphaeriaceae

Calosphaeria minima Tul. On *Cornus alternifolia*, Upper Brookside, July 15, 1931 (1055).

Various similar fungi are undoubtedly placed under this binomial. This collection shows numerous, small, angular, brown pustules with a disk of a few irregular, somewhat elongate ostioles, with no ectostroma. Asci numerous, clavate, short-stalked, persistent in the hymenium, eight-spored, $22-26 \times 3.5-4 \mu$. Spores allantoid, or inequilateral, one-celled, $4-5.5 \times 1-1.5 \mu$. Paraphyses rather broad, tapered above, longer than the asci.

Xylariaceae

Daldinia concentrica Ces. and de Not. On *Alnus* spp., Economy, Colchester Co., July 9, 1926 (94); Green Oak, Colchester Co., June 12, 1929 (221); Salmon River, July 14, 1931 (1019).

Hypoxylon coccineum Bull. On *Fagus grandifolia*, Economy Lake, June 16, 1926 (318a); Mt. Thom, Aug. 10, 1931 (318b).

Hypoxylon cohaerans Fr. On *Fagus grandifolia*, Folley Lake, Sept. 3, 1928, leg. A. R. Prince (6074); Upper Brookside, June 29, 1931 (326).
Common on dead or injured beech, forming irregular black stromatic crusts.

Hypoxylon fuscum Fr. On *Alnus* sp., Rifle Range, Truro, June 25, 1926 (P385a); Deep Hollow Rd., Wolfville, June 25, 1926 (P384); on *Corylus cornuta*, Upper Brookside, July 29, 1931 (446).

Hypoxylon Morsei B. and C. On *Alnus* sp., and *Salix* sp., Victoria Park, July 23, 1931 (1123 and 1128).

Hypoxylon multifforme Fr. On *Betula papyrifera*, Mill Lake, Upper Musquodoboit, Halifax Co., June 14, 1925, leg. A. R. Prince (102); Killag Mines, July 30, 1931 (1189).
Common on birch, usually as reddish fusoid stromata erumpent through laterally elongate ruptures of the periderm.

Hypoxylon rubiginosum (Pers.) Fr. On *Acer spicatum*, New Glasgow Rd., July 25, 1931 (1149 and 1153); Folley Lake, Aug. 24, 1931; Upper Brookside, July 18, 1929 (137); on decorticated wood, Middle River, Victoria Co., Aug. 5, 1931 (1226); Mt. Thom, Aug. 10, 1931 (1251).

Quite common and variable in form.

Hypoxylon ustulatum (Bull.) Fr. (*Ustulina vulgaris* Tul.). On *Betula* sp., Mill Lake, Upper Musquodoboit, Halifax Co., June 14, 1925, leg. A. R. Prince (1134); on mossy log, Middle River, Victoria Co., Aug. 5, 1931 (1228).

Xylaria castorea Berk. On decayed wood of various species, Princeport, Sept., 1927 (6107); Folley Lake, Aug. 23, 1927 (6042); Upper Brookside, Sept. 28, 1926 (6050); Oct. 20, 1926 (103); July 4, 1931 (401).

The first three collections were made by A. R. Prince.

Xylaria coprophila sp. nov. (Figs. 17, 18).

Stromata brown-black, 0.5–1.5 cm. tall. Stalk terete to flattened, 0.5–1 mm. in diameter, roughened with longitudinal wrinkle-like ridges; not tomentose. Fertile head flattened, with a pointed apex which remains sterile, 1–1.5 mm. in diameter. Perithecia $250-300 \mu$ in diameter, scattered or densely crowded, base partially sunken in the surface of the stroma or almost superficial. Ostiole rather prominent, conic-papillate. Perithecial wall thin membranous. Asci cylindric, short stipitate, spore-bearing portion $60-70 \times 5-5.5 \mu$; stipe $15-20 \mu$ long. Spores uniseriate, inequilateral, one-celled, dark brown, $9-10.5 \times 3.5-4.5 \mu$.

Type: Herbarium L. E. Wehmeyer, on porcunium dung, New Glasgow Rd., Pictou Co., N.S., VIII, 1931, leg. A. H. Smith (No. 1490).

Material of this collection was sent to Dr. J. H. Miller, who states (in litt.) that it is different from any species known to him on this substrate. The description of *X. graminicola* Ger. fits this material in many respects. Doctor Linder kindly compared this collection with the type of that species in the Farlow Herbarium and reports that *X. graminicola* is distinct in the presence of a dark brown tomentum on the stem, more strongly inequilateral spores, and several other minor differences.

Xylaria coprophila sp. nov. Stromata in stercorebus *Hystrix*, 0.5–1.5 cm. alta, stipite tereti vel compresso longitudinaliter costato, glabro; capite fertili depresso, 1.0–1.5 mm. diametro, apice cacumine sterili praedito; peritheciis $250-300 \mu$ diametro, dispersis vel

dense aggregatis basi in stromate demidio immersis vel fere superficialibus; ostioliis prominulis conice papillatis; membrana peritheciali tenui; ascis cylindricis, breviter stipitatis, parte sporifera 60–70 μ longis, 5–5.5 μ latis; stipite 15–20 μ longo; sporis uniseriatis, inaequilateralibus, 1-cellulis, atrobrunneis, 9–10.5 μ longis, 3.5–4.5 μ crassis.

Specimen typicum in auctoris herbario, legit A. H. Smith (No. 1490) in fimis hystericinis, prope "New Glasgow Rd.," in Nova Scotia, VIII, 1931.

Xylaria Hypoxylon (L.) Grev. On decayed wood, mossy logs, etc., Upper Brookside, Aug. 21, 1931 (1418); Wentworth Valley, Cumberland Co., Aug. 29, 1931, leg. A. H. Smith (1454); Salmon River, Aug. 21, 1931 (1679).

Xylaria polymorpha (Pers.) Grev. On *Picea* sp., Salmon River, near Truro, Oct. 2, 1925, leg. A. R. Prince (6270).

Dothideales

Diobotryon morbosum (Schw.) Theiss. and Syd. On *Prunus virginiana* L., Truro, July 4, 1925, leg. A. R. Prince (1103).

Dothidella Kalmiae (Pk.) Sacc. On *Kalmia angustifolia*, Oldham, Halifax Co., Sept. 5, 1929 (265); Portapique Beach, Aug. 8, 1933 (1621).

This fungus causes a witches' broom effect with stout elongate upright branches bearing dwarfed leaves. These branches are heavily blackened as the stroma develops over the entire surface. Later the minute points of the ostioles and the papillate swellings of the protruding perithecial locules appear. At the time of these collections, the current year's stromata were immature and the previous year's were old and mostly empty. The only spores seen in them were fusoid, hyaline, two-celled, 12–17 \times 2–3 μ , and it is not certain that these were not of some parasitic pyrenomycete, although they were seen in apparently normal stromatic locules. Peck gives the spores as unequally two-celled, 10–12 \times 5–6 μ , but the gross appearance is the same.

Dothidella Osmundae (Pk. and Clint.) Sacc. On petioles of *Pteritis nodulosa* (Michx.) Niewl., Upper Brookside, July 15, 1929 (15); Aug. 12, 1931 (1664); on *Pteridium aquilinum*, Upper Brookside, July 27, 1929 (141); on *Osmunda cinnamomea* L., Upper Brookside, July 8, 1931 (445).

Endodothella Junci (Fr.) Theiss. and Syd. On *Juncus* sp., Upper Brookside, July 4, 1931 (399); Deep Hollow Rd., Wolfville, June 25, 1926 (107).

Phyllachora Wittrockii (Erikss.) Sacc. On *Linnaea borealis* L. var. *americana* (Forbes) Rehd., Victoria Park, June 4, 1931 (403); July 22, 1933 (1555).

This is a very striking parasite. The upright tips of *Linnaea* are attacked and the entire stem for a distance of one to two inches is surrounded by a blackened pseudo-parenchymatous stroma 300–500 μ in thickness. This stroma is at first wrinkled and then punctate with the minute ostiolar openings. All of the collections made contained only immature perithecial locules without spores. The ascospores apparently mature late in the fall, or, more likely, early spring. The young stromata are covered with a grayish bloom which is the conidial stage consisting of numerous stout, spine-like, pointed conidiophores which bear at their apex a single ellipsoid, one-celled, hyaline to pale brown conidium, 9–9.5 \times 3 μ .

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PROTOPLASMIC CONTINUITY IN THE POWDERY MILDEW *ERYSIPHE GRAMINIS* DC.¹

BY HAROLD J. BRODIE²

Abstract

The protoplasm in the mycelium and conidiophores of the barley mildew (*Erysiphe graminis* DC.) is continuous from cell to cell. Each transverse septum is provided with a minute central pore through which passes a delicate strand of cytoplasm 1–1.5 μ in width. The cytoplasmic strands and the septal pores have been readily demonstrated by means of a slight modification of Wahrlich's technique, and their presence recorded by means of photomicrographs and drawings. The cytoplasmic connections have also been seen in living unstained mycelium.

Streaming of cytoplasm from cell to cell was not observed in the present study, possibly because of the slow rate of flow or because of the difficulty of examining the mycelium without disturbing it.

Introduction

The presence of perforations in the septa of the conidiophores of *Erysiphe Polygoni* DC., and the continuity of the protoplasm through the perforations were reported recently in a paper by Brodie and Neufeld (1). In the young conidiophore of that fungus, the transverse wall appears as a ring of shiny material which grows toward the inside until a disk-like septum is formed. In the centre of the disk, a hole is left through which passes a fine thread of protoplasm. There is, therefore, continuity of protoplasm from the base of the conidiophore to the apex. Pores and protoplasmic threads were also observed in the mycelium.

Special attention was not given to this matter by Brodie and Neufeld, inasmuch as it was felt that Buller's treatment (2) of the subject of protoplasmic continuity in fungi had established the essential facts. Demonstration of septal pores in the Erysiphaceae merely added one more group to a long list covering most of the groups of fungi³.

However, subsequent studies of the conidiophores and mycelium of *Erysiphe graminis* DC. have shown that this fungus, like *E. Polygoni*, is a favourable subject for the demonstration of septal pores and protoplasmic continuity. By the use of a slight modification of Wahrlich's (6) technique (to be described in detail later in this paper), it was found possible to obtain excellent preparations showing the continuity of protoplasm, through septal pores, from cell to cell in chains of conidia and from cell to cell in the mycelium.

The purpose of this paper is to establish the existence of septal pores and protoplasmic continuity in *Erysiphe graminis* by means of photomicrographs

¹ Manuscript received July 30, 1942.

Contribution from the Department of Botany, University of Manitoba, Winnipeg, Man. This research was carried out with financial assistance received from the National Research Council of Canada.

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³ Pores in the septa of mycelium of *Sphaerotheca Castagnei* Lev. (*Erysiphe Cichoracearum* DC.) were observed by P. A. Dangeard in 1897 (*Botaniste (Sér. 5)*, 5 : 255. 1897).

and drawings. This has seemed especially desirable because the question as to whether septal pores and protoplasmic bridges actually exist in the higher fungi has been raised by such workers as Noble (4, 5) who reported being unable to find pores in the septa of the mycelium of *Typhula Trifolii* Rostr.

Material and Methods

The fungus used in this investigation was *Erysiphe graminis* DC., which appeared on barley plants in the greenhouses of the University of Manitoba in December, 1941. It was transferred to the variety of barley, O.A.C. 21, and cultures were maintained on young barley plants inoculated by shaking spores from an older culture on to seedlings 3 in. high. Infected plants were kept on the greenhouse bench in full light. The fungus was usually at its best for study about a week or 10 days after inoculation.

Wahrlich's technique for demonstrating septal pores and protoplasmic continuity has been described by Buller (2, pp. 89-91). It consists of fixing the living fungus in a watery solution of iodine in potassium iodide, treating with chlor-zinc iodine to cause the walls of the fungus to swell, and staining with a strong solution of iodine in potassium iodide.

Difficulty was experienced at first in obtaining good fixation. Shrinkage caused the thin thread of protoplasm connecting adjoining cells to break. After many trials, the most satisfactory concentration for good fixation was found to be that made by diluting the strong iodine solution about 1 : 20. The exact proportions used are given herewith.

Strong iodine in potassium iodide solution:

Iodine	3 gm.
Potassium iodide	3 gm.
Distilled water	20 cc.

This strong stock solution was used: (1) for making the dilute fixative and (2) for staining the protoplasm.

The dilute fixative was made by adding 10 drops of the strong iodine in potassium iodide solution to 20 cc. of distilled water.

The complete schedule follows:

1. Fix fresh material in *diluted* iodine in potassium iodide for two hours.
2. Rinse with distilled water.
3. Add chlor-zinc iodine to material on slide. (It may be necessary to heat cautiously until steam appears.)
4. Remove chlor-zinc iodine with blotting paper and rinse with water.
5. Add several drops of strong iodine in potassium iodide and allow to stand 30 min.
6. Remove strong solution and rinse quickly with dilute solution.
7. Mount in chlor-zinc iodine, or in 30% glycerine.

Nigrosin was also used as a stain (10% aqueous) and, although it did not reveal any more detail than did the iodine stain, it was found more satisfactory in making preparations to be photographed.

General Appearance of Preparations

Using the method described above, the mycelium, conidiophores, and conidia of *Erysiphe graminis* presented the following appearance.

The lateral walls and septa were light yellow, highly refractive, and swollen. The septa appeared more swollen than the lateral walls and often became six to eight times their original thickness (Fig. 7). The extent to which the walls were swollen varied considerably in different preparations. Prolonged heating would cause great swelling but did not improve the appearance of protoplasmic connections.

The degree of plasmolysis of the protoplasts varied from very slight (Figs. 10, 11) to severe (Figs. 12, 13). Where plasmolysis had been severe, the protoplasmic connection between cells was usually broken (Fig. 8a).

When strong iodine in potassium iodide was used as the stain, the protoplasts were stained deep brown, almost black. Within them, various granules could be seen, and frequently a conidium was found containing a single stained nucleus (Fig. 11). Staining of the nuclei was more common when nigrosin was used (Fig. 6).

In none of the preparations was it possible to discern the double nature of the wall suggested by the drawings from Wahrlich's paper that were reproduced by Buller (2, pp. 90 and 94).

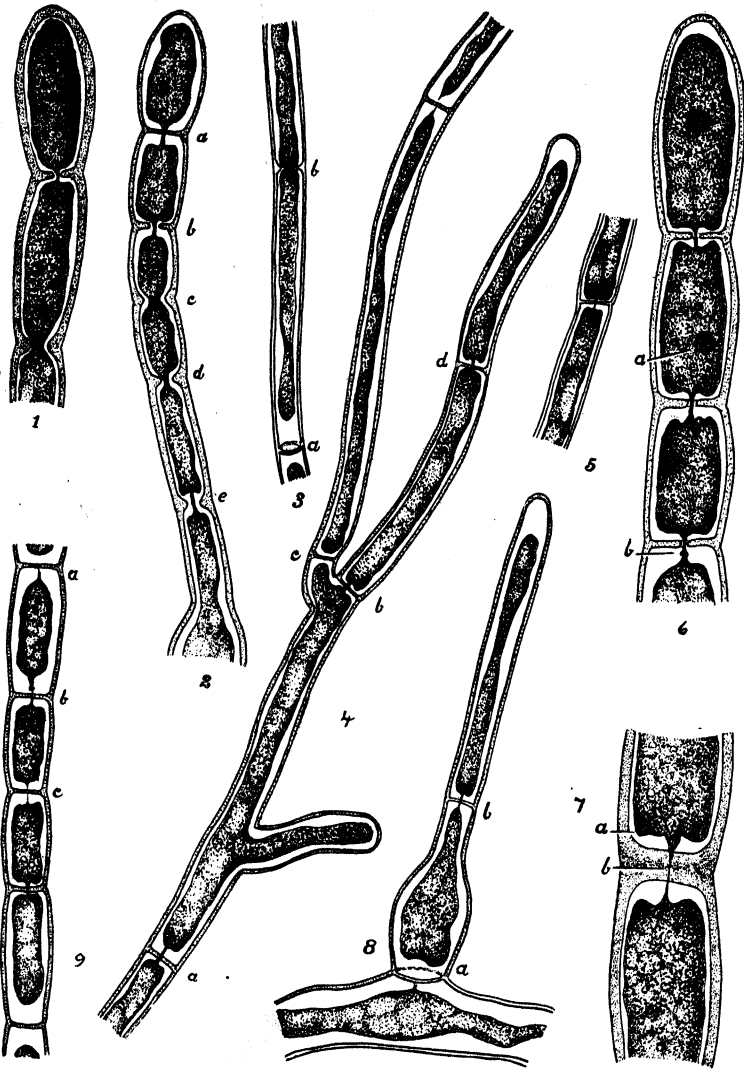
The Septal Pore and Protoplasmic Connections

In *Erysiphe graminis*, the protoplasts in living, actively growing mycelial cells and conidial cells in chains are connected by thin strands of protoplasm. When the septum between two cells is young, the protoplast is but slightly constricted by the septum (Fig. 2c and Fig. 3b); but, as the ring of wall material gradually closes, the protoplasmic connection between cells is reduced to a very thin thread of cytoplasm between 1 and 1.5 μ thick. Sometimes the thread appears homogeneous, at other times granular. Frequently there is a knot on the cytoplasmic strand (Fig. 11, a, b, and Fig. 6b). Even where severe plasmolysis has caused the thread to be broken, traces of it can frequently be seen at the ends of the protoplasts as shown in Fig. 9, a, c.

Connections are present between mycelial cells everywhere in the mycelium, from growing hyphal tips (Fig. 4d) back into older mycelium (Fig. 5). They are harder to demonstrate in older mycelium, however, where they frequently become broken between highly vacuolate cells.

In *Erysiphe graminis*, each chain of conidia arises from a large bulbous basal cell. The protoplasmic connection between this cell and the mycelium below it is usually very conspicuous, the thread being long. This connection is illustrated in the photomicrograph, Fig. 14.

It was surprising to find that protoplasmic connections are almost invariably present between adjacent conidia in chains. Often a certain conidium in a chain (e.g. b in Fig. 15) would appear to lack connection with its fellows, but



Erysiphe graminis DC. All figures except Fig. 6 are from material stained with iodine.

FIG. 1. Apex of chain of conidia, showing much swollen walls and protoplasmic connections between spores. $\times 1000$. FIG. 2. Chain of conidia showing protoplasmic continuity from bulbous basal cell to terminal conidium: a, transverse septum scarcely swollen; b to e, transverse septa swollen in varying degrees. $\times 830$. FIG. 3. Hypha near growing tip, showing fully formed septum at a (the pore not evident) and protoplasm severed by extreme contraction, and young septum at b. $\times 645$. FIG. 4. Mycelium showing protoplasmic connections at a, b, and d; protoplasmic continuity disrupted at c by contraction. $\times 830$. FIG. 5. Distinct protoplasmic thread between two cells in old part of mycelium. $\times 830$. FIG. 6. Chain of conidia stained with nigrosin showing protoplasmic connections, nucleus at a, and knot on protoplasmic thread at b. $\times 1200$. FIG. 7. Connection between two conidia at b, and lobed appearance of end of protoplast at a. $\times 1330$. FIG. 8. Young conidiophore before spore formation; at a, protoplasmic connection with bulbous basal cell broken by contraction; clearly visible. $\times 830$. FIG. 9. Chain of conidia showing protoplasmic threads: at a and c, threads partly ruptured by contraction. $\times 830$.

always connections were found between other conidia in the same chain. The apparent lack of protoplasmic connections in these examples is probably to be explained by shrinkage due to imperfect fixation, with consequent rupture of the delicate thread connection.

In *Erysiphe Polygoni*, protoplasmic continuity is broken between the terminal mature conidium and the conidiophore, and the pore in the septum is plugged some time before the spore is shed (1). No evidence of plugging of the septum of the terminal conidium in *E. graminis* has been found in the course of the present investigation. Protoplasmic continuity is maintained even into the terminal conidium (e.g. Figs. 1, 2, 6).

The pore in the septum has only rarely been seen directly, although its presence is indicated beyond all doubt by the continuity of protoplasm through the septum. In a few preparations, the pore was seen directly, usually in hyphae or conidiophores that lay at such an angle that the microscope looked down on the face of the septum rather than on the edge. The photograph, Fig. 12, was made from a hypha in which a pore was particularly clearly seen at *a*. The hypha lay at a slight angle and, although the photograph leaves much to be desired, the pore in the centre of the septum is fairly well shown. The septum was near the growing tip of a hypha, and the pore is therefore larger than is found in fully formed septa.

Slight variations in optical conditions made a great difference to the appearance of the septum in preparations. Usually it appeared as a relatively thick unbroken line (Fig. 4). Frequently, however, it appeared broken (Fig. 2), especially where the walls had been greatly swollen. Whether the septum had the appearance of being broken or not, the protoplasmic thread was always continuous from one protoplast to the next, unless it had itself been broken by shrinkage. There does not seem to be any room for argument that because protoplasts were occasionally seen with no protoplasmic bridge between them (because of imperfect fixation), there is no pore in the septum. In fact, pores were occasionally seen even where no protoplasmic connection could be found.

A peculiar feature of the stained preparations which may be noted is the lobed appearance of the protoplasts adjacent to the septa (Fig. 7 and Fig. 15, *a*, *b*). The same feature can be seen in Wahrlich's illustrations reproduced by Buller (2, p. 90).

The photographs reproduced in Plate I were all taken with an oil immersion lens (Zeiss, N.A. 1.3) which had a shallow focus. It was therefore difficult to photograph protoplasmic threads that lay at different depths in a chain of cells. For example, the apparent lack of protoplasmic threads at *a* in Fig. 16, and at *b* in Fig. 15, is due solely to the threads being quite out of focus.

Although it may be thought that a treatment of delicate protoplasmic structures that involves heating with chlor-zinc iodine might produce artifacts, the fact is that it is possible to see the protoplasmic connections which have been described in *untreated* mycelium and conidial chains of *Erysiphe graminis*.

Several times, *living* mycelium and conidial chains were examined when protoplasmic bridges between conidia in chains were seen clearly with the oil immersion lens. There can therefore be no doubt about the existence of protoplasmic continuity between cells in this fungus.

As a warning to those who may wish to employ Wahrlich's technique for the demonstration of protoplasmic threads and septal pores in other fungi, it may be well to re-emphasize the necessity of obtaining good fixation. Fixatives other than that used in the present investigation might prove more satisfactory. It is essential that no marked contraction of the protoplasm take place in order that the delicate strands that pass through the pores remain intact. At the outset, the writer had to find by trial the dilution of iodine fixative that gave the best results, and the conditions for fixation and staining may be expected to be slightly different in different fungi.

Search for Protoplasmic Streaming

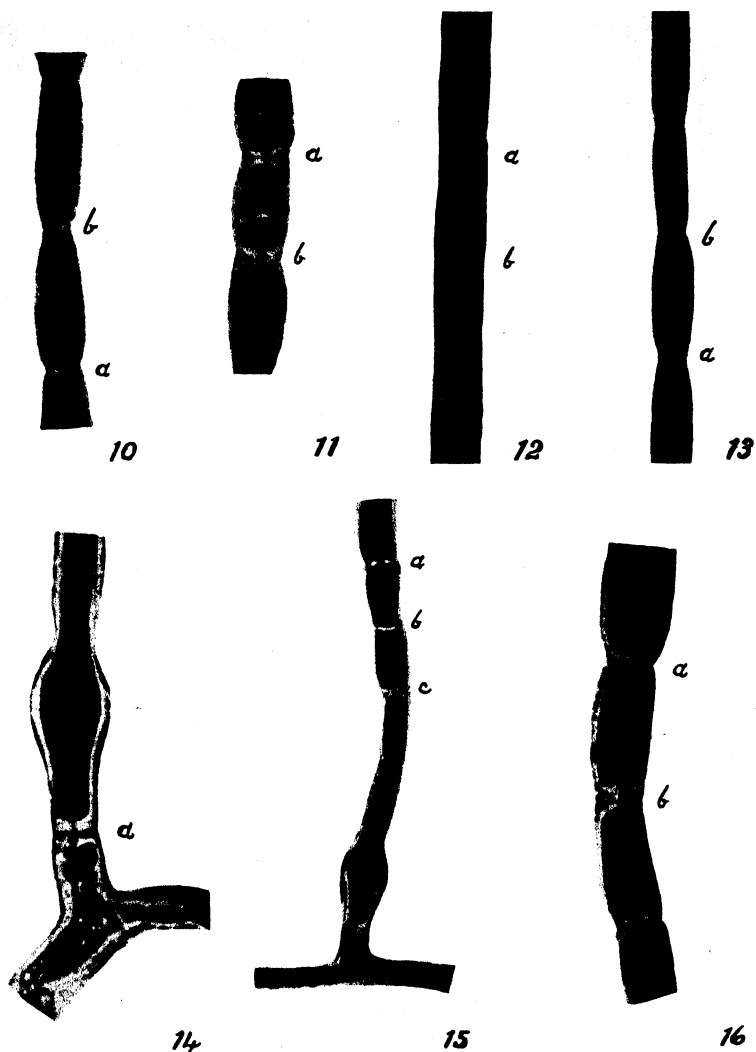
It was pointed out by Brodie and Neufeld (1) that septal pores and protoplasmic continuity are very important in the Erysiphaceae. Since the haustoria absorb the food material, and since the mycelium and conidiophores are aerial, food materials and protoplasm must pass from the haustoria to the aerial conidiophores via the pores in the transverse septa of the mycelium.

The streaming of cytoplasm from cell to cell has been demonstrated for a number of fungi by Buller (2), Dowding (3), and by others, and might be expected in the mycelium and conidiophores of *E. graminis*. Brodie and Neufeld (1) reported being unable to observe any flow of cytoplasm in living mycelium of *E. Polygoni*, and the writer therefore made a special effort to observe cytoplasmic streaming in *E. graminis*.

Patient and exhaustive study of all parts of the mycelium and conidiophores failed to reveal any continuous movement of cytoplasm of living material of *E. graminis*. At first, mycelium was carefully removed from infected leaves with needles and mounted in water for examination. Later, in order to avoid (as far as possible) injury to the mycelium, pieces of barley leaves infected with mildew were gently pulled apart lengthwise with the purpose of exposing, between the long strips of leaf, bits of mycelium that had not been greatly disturbed. In some examinations, the mycelium was mounted in liquid petrolatum instead of water. The cytoplasm could be clearly seen in all preparations. It is very granular and it should be possible to see streaming if it takes place.

Occasionally a slight movement was observed, but this was brought about by rupture of a hypha or by a sudden shift in the position of vacuoles. At no time was cytoplasmic streaming observed, although the search was carried on for several weeks in material of various ages.

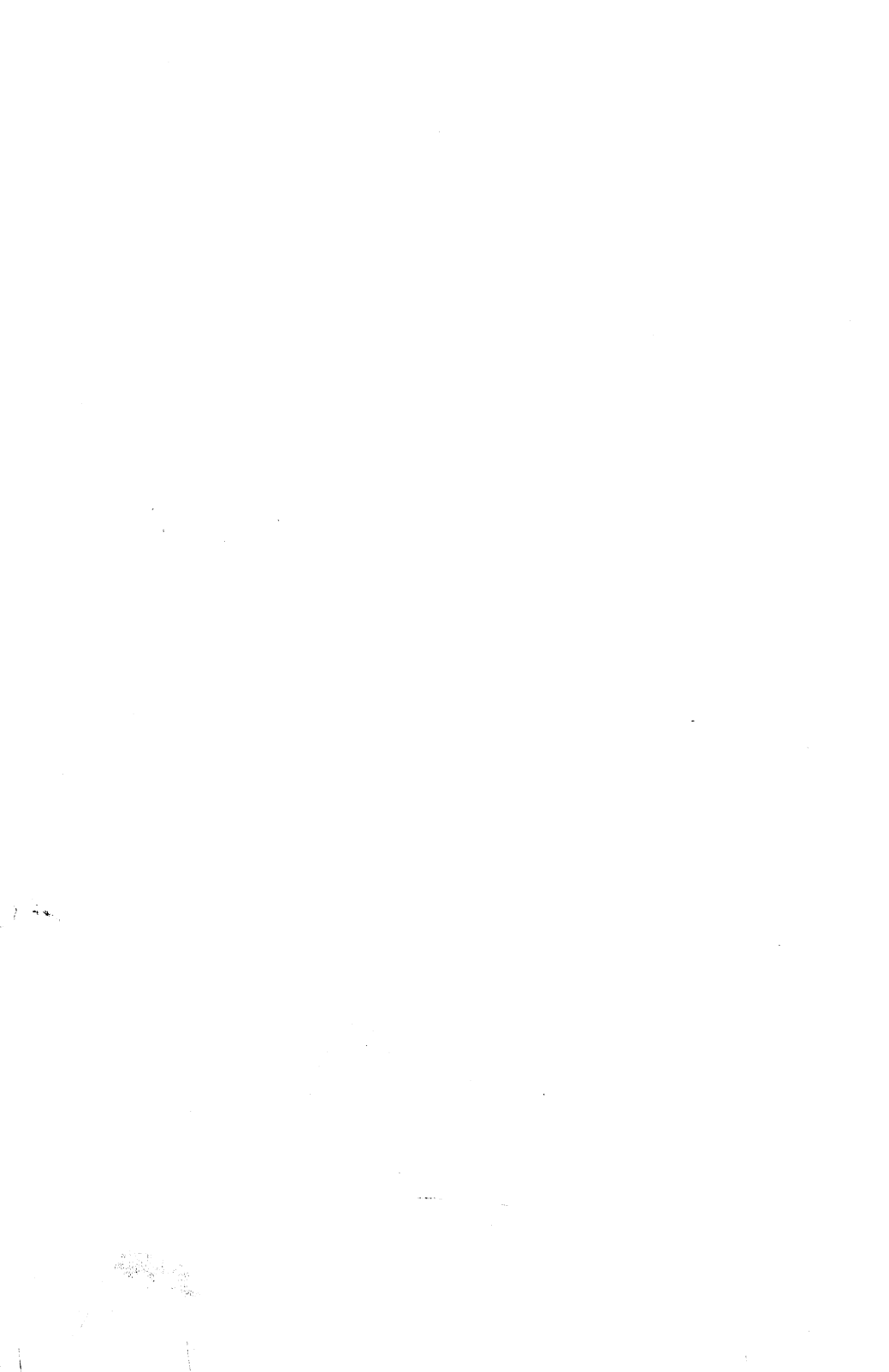
It is most improbable that no streaming of cytoplasm at all occurs in the mildew mycelium. The mycelium grows very slowly, and failure to observe streaming by direct observation may be due to the slow rate at which it takes



EXPLANATION OF PLATE

Erysiphe graminis DC. Photomicrographs of material stained in nigrosin, except Figs. 10, 11, and 12 which are of material stained in iodine. All photographs untouched except for blocking out of background.

FIG. 10. Two conidia near end of chain showing connection at b; connection at a broken by shrinkage. $\times 660$. FIG. 11. Three conidia showing connections at a and b. $\times 830$. FIG. 12. A bit of mycelium near growing end showing a connection at b; at a, a septum is seen thrown slightly out of focus to show the pore in the centre. $\times 660$. FIG. 13. Chain of conidia with connecting threads at a and b. $\times 580$. FIG. 14. Connection between basal cell of chain of conidia and mycelium below, shown at a. $\times 830$. FIG. 15. Conidia with connections at a and c; at b, the connection was present but completely out of focus. $\times 410$. FIG. 16. Chain of conidia showing protoplasmic bridge at b; a thread was also visible at a in the material but it is not in focus in the photograph. $\times 830$.



place. It may also be due to the difficulty of examining the mycelium without disturbing it.

Acknowledgment

This research has formed part of a project for which financial assistance was secured from the National Research Council of Canada, to which the writer wishes to acknowledge his indebtedness.

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A STAINING TECHNIQUE FOR EVALUATING THE TOXICITY OF AN ANTIBIOTIC SUBSTANCE OF MICROBIOLOGICAL ORIGIN¹

BY H. KATZNELSON²

Abstract

A method is described in which the absorption of neutral red by dead cells of *Schizosaccharomyces pombe* is used as an index of the toxicity of an antibiotic agent produced by a bacillus.

Progress in research work with antibiotic substances produced by micro-organisms is frequently delayed because a rapid method for determining the toxicity of these agents is lacking. The most common procedures for evaluating the activity of these substances utilize their ability to inhibit, dissolve, or kill the test organism (1, 4, 5, 7). These techniques often involve standardization of bacterial suspensions, plating, incubation for a period of 24 or 48 hr., and counting of plates; a study of the influence of these toxic principles on fungi not infrequently involves incubation for two to seven days before satisfactory results are obtained (2). It is obvious that a procedure that will eliminate such a step as a long incubation period alone, and yield results within two or three hours is highly desirable.

It was recently reported (2) that the yeast *Schizosaccharomyces pombe* was one of many fungi inhibited (and killed) by a thermostable substance elaborated by an aerobic sporeforming bacillus. This yeast was found by Knaysi (3) to take up neutral red on dying; the cells usually stained a deep red but even faintly stained cells were shown to be dead whereas living, healthy cells remained colourless. The present paper reports an attempt to utilize this phenomenon in various experiments with the toxic agent.

Description of the Method

The yeast was grown on nutrient agar containing 1% peptone and 2% dextrose for 24 hr. at 28° C. after which the cells were suspended in potato dextrose broth at pH 6.3; the suspension was standardized by means of a counting chamber so that one ml. contained 160,000,000 cells. To one ml. of the toxic test material in a small glass tube ($2\frac{1}{2} \times \frac{3}{8}$ in.) were added 0.5 ml. of the cell suspension and 0.5 ml. of a 1 : 5000 aqueous solution of neutral red; the tube was plugged, shaken thoroughly, placed in a water-bath maintained at 36° C. for three hours, and agitated intermittently. On removal from the water-bath the tube was again shaken and some of its contents transferred to a counting chamber. Counts were made of the total number

¹ Manuscript received September 14, 1942.

Contribution from the Department of Bacteriology, Cornell University, Ithaca, N.Y., and the Division of Bacteriology and Dairy Research, Science Service, Department of Agriculture, Ottawa. (Journal Series No. 158.)

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and the number of stained, shrunken organisms in 20 of the smallest squares and the results were expressed as percent stained cells. When quantitative data were not required the procedure was expedited by placing a drop of the suspension in the test-tube on a slide, examining it under the microscope, and estimating roughly the percentage of stained cells in several fields. The accompanying photographs (Fig. 1) indicate that differences between stained and unstained cells are readily apparent, a fact that facilitates the quantitative or semiquantitative determination of the toxicity of the antibiotic substance.

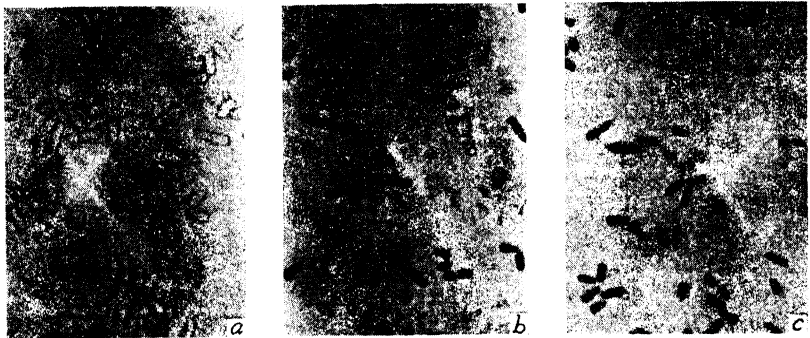


FIG. 1. Influence of toxic medium on *S. pombe* as indicated by neutral red staining; (a) check; (b) 50% toxic medium; (c) 100% toxic medium. ($\times 500$).

The final concentration of neutral red in the incubating mixture is 1 : 20,000 and is not harmful to the yeast. The reaction of the test and suspending fluids should be slightly acid (pH 6.0 to 6.5) as neutral red becomes yellowish and useless in an alkaline medium. Various suspending solutions (buffers) for the yeast cells were tested and potato dextrose peptone broth was selected (Table I).

The graphs in Fig. 2 are illustrative of the results of many experiments to determine the conditions that would favour the greatest number of cells being

TABLE I

STAINING OF *Schizosaccharomyces pombe* IN THE PRESENCE OF A TOXIC AGENT AS AFFECTED BY DIFFERENT BUFFER SUSPENDING SOLUTIONS

	Suspending fluids, pH 6.3					
	Borate	Citrate	Glycine	Phosphate	Phthalate	Potato dextrose broth
	Stained cells, %					
Controls	1.0	20.0	1.0	0.1	0.1	0.2
Toxic substance added	27.0	35.0	25.0	34.0	21.0	89.0

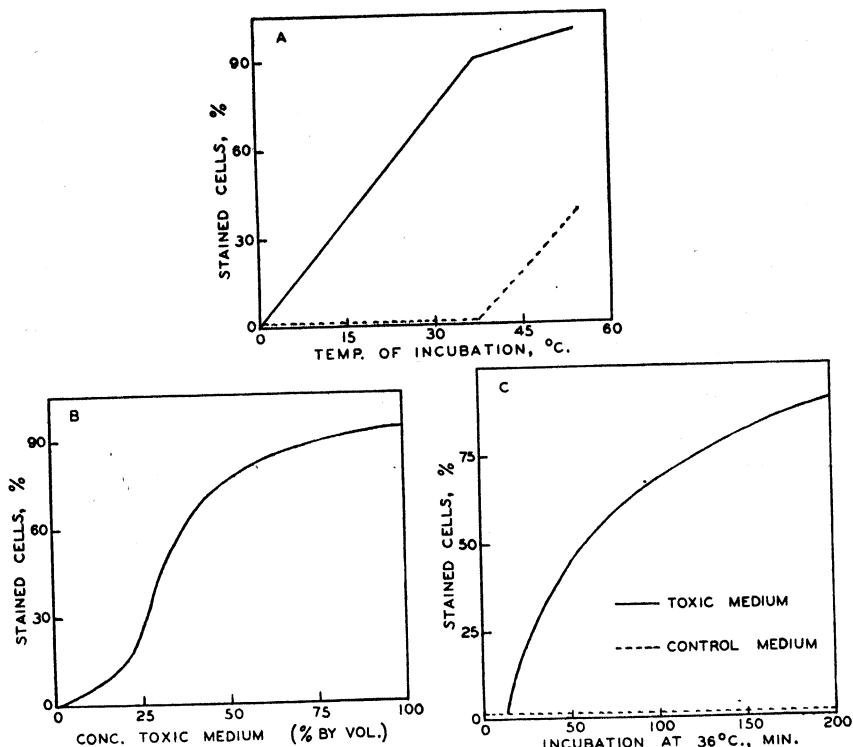


FIG. 2. Influence of temperature of incubation, concentration of toxic medium, and period of incubation on the staining of *Schizosaccharomyces pombe* by neutral red.

killed by the agent. In the trials represented by Graphs A and C, undiluted toxic medium (2) was used. A temperature of 36°C. and an incubation period of three hours were finally selected (a considerable number of cells were killed in the control series at 55°C.). Graph B indicates the effect of different concentrations of toxic medium on the yeast. The most effective range appears to be between 15 and 50% (by volume) of toxic medium.

In the numerous studies with the toxic principle the above technique proved a rapid, useful tool. Concurrently, however, the fungus *Rhizoctonia Solani* was also used as an index of the potency of the inhibiting substance for comparative purposes. The following experiments illustrate the applicability of this staining procedure.

As previously reported (2) toxic medium is thermostable in neutral or slightly acid solutions, loses its potency when heated in a strongly alkaline medium, but retains it to some extent in strongly acid solutions. In these tests, toxic and potato dextrose media were adjusted to pH < 1 with hydrochloric acid and pH > 12 with sodium hydroxide and heated 15 min. in boiling water; tubes with the above media kept at pH 6.0 were also included in this series.

After heating, the pH of all the tubes was brought to 6.0 and all fluids including unheated toxic and potato dextrose media were tested by the yeast staining method and by the dilution method with *R. Solani*; the results are presented in Table II. Both methods bear out the observation regarding the influence of heating toxic medium in acid and alkaline solutions.

TABLE II

INFLUENCE OF HEAT ON TOXIC MEDIUM IN ACID AND ALKALINE SOLUTIONS AS DETERMINED BY YEAST STAINING AND INHIBITION OF *Rhizoctonia Solani*

Test method		Treatment of medium							
		Unheated		Heated					
				pH < 1		pH > 12		pH 6.0	
		T.M.†	P.D.B.††	T.M.	P.D.B.	T.M.	P.D.B.	T.M.	P.D.B.
Yeast staining*		90	2	39	1	1	1	92	1
Inhibition of <i>R. Solani</i> **	Conc. of test material (% by vol.)								
	0	4	4	4	4	4	4	4	4
	1	4	4	4	4	4	4	4	4
	3	2	4	4	4	4	2	4	4
	5	1	4	4	4	4	0	4	4
	7.5	0	4	3	4	4	4	0	4
	10	0	4	2	4	4	4	0	4
	15	0	4	0	4	4	4	0	4
	25	0	4	0	4	4	4	0	4
	50	0	4	0	4	1	1	0	4

* % Stained cell.

** Good growth = 4; no growth = 0.

† T.M. = toxic medium.

†† P.D.B. = potato dextrose broth.

In an experiment to determine the influence of pH of medium on the production of toxic principle by the bacillus a range from pH 4.4 to 7.5 was used. Flasks at different pH levels were inoculated and incubated for seven days after which the pH was adjusted to 6.0, the medium diluted with two volumes of potato dextrose broth and tests made using the yeast technique. Apparently an acid reaction favours the production of the inhibitory agent (Table III).

TABLE III

ELABORATION OF TOXIC MATERIAL AT VARIOUS pH LEVELS

pH of medium	4.4	4.8	5.5	5.9	6.2	6.7	7.1	7.5
Stained yeast cells, %	59	60	35	36	35	21	20	13

Agitation of the culture medium is frequently employed to stimulate the activity of certain organisms (6) but it was deleterious in this instance (Table IV).

TABLE IV

EFFECT OF AGITATION OF CULTURE MEDIUM ON PRODUCTION OF TOXIC MATERIAL AS DEMONSTRATED BY YEAST STAINING

Concentration of medium tested (% by volume)	Treatment of medium			
	Continuous agitation for 1 week		No agitation	
	Culture medium	P.D.B. control	Culture medium	P.D.B. control
	Stained cells, %			
0	0	0	0	0
25	0	0	35	0
50	0	0	60	0
100	0	0	100	0

The composition of the culture medium exerts a decided influence on the elaboration of inhibitory material. With potato dextrose broth as base, varying amounts of peptone were added and the resulting culture media were tested with both methods (yeast staining and inhibition of *R. Solani*). Both procedures indicate that low concentrations of peptone (resulting in poor growth of the bacillus) are unfavourable for the production of the toxic substance (Table V).

TABLE V

STAINING OF *S. pombe* AND INHIBITION OF *R. Solani* BY TOXIC MATERIAL PRODUCED IN POTATO DEXTROSE BROTH CONTAINING VARIOUS AMOUNTS OF PEPTONE

Test method		Peptone, %						
		0.05	0.1	0.5	1.0	1.5	2.0	2.5
Yeast staining, %		10	15	53	74	86	88	90
Inhibition of <i>R. Solani</i> *	Conc. test material (% by volume)							
	0	4	4	4	4	4	4	4
	1	4	4	3	3	3	3	3
	3	3	2	1	0	0	0	0
	5	2	2	1	0	0	0	0
	7.5	2	1	0	0	0	0	0
	10	1	1	0	0	0	0	0
	15	1	0	0	0	0	0	0

* Good growth = 4; no growth = 0.

By similar methods it was found that low concentrations of dextrose or sucrose were also unfavourable, the optimum concentration being 2.5%. Sucrose was slightly less favourable than dextrose (Table VI). The same trend was observed with the *R. Solani* technique.

TABLE VI
EFFECT OF CONCENTRATION OF GLUCOSE AND SUCROSE ON YIELD OF TOXIC MATERIAL

Sugar used	Sugar concentration, %					
	0.1	0.5	1.0	1.5	2.0	2.5
	Stained cells, %					
Dextrose	30	60	60	68	81	92
Sucrose	32	49	58	57	60	72

Charcoal was found to remove the toxic substance from the culture medium (2). Again the yeast staining and *R. Solani* methods bear this out (Table VII).

TABLE VII
ADSORPTION OF TOXIC MATERIAL BY CHARCOAL

Test method		Treatment of material			
		Toxic medium		Potato dextrose broth	
		Untreated	Charcoal treated	Untreated	Charcoal treated
Yeast staining, %		90	1	1	1
Inhibition of <i>R. Solani</i> *	Conc. test material (% by volume)				
	0	4	4	4	4
	1	1	4	4	4
	2	1	4	4	4
	3	0	4	4	4
	5	0	4	4	4
	10	0	4	4	4
	25	0	4	4	4
	50	0	4	4	4
	75	0	4	4	4
	100	0	4	4	4

* Good growth = 4; no growth = 0.

The above experiments suffice to indicate the usefulness of the yeast staining technique for studying the lethal nature of an antibiotic agent of microbiological origin. It may also find application in evaluating the fungicidal or bactericidal property of a wide variety of commercial disinfectants and chemotherapeutic agents.

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12.1.60		
25.3.60		
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23.7.60		
3.2.66		
16.1.67		
15.3.67		
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